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The purpose of this project is twofold. First, to construct novel bispecific antibodies that can be expressed as a single-chain in *E. coli*.. Second, to develop an *in vivo* animal model, using a TCR transgenic/RAG$^{-/-}$ strain of mice, for evaluating agents that will redirect the activity of T cells. In the past year, the following specific aims toward these goals were accomplished: 1) Two different scFv$_2$ antibodies were expressed as inclusion bodies in *E. coli*, purified and tested *in vitro*; because these showed less than optimal activity (>10 nM), one of the scFv$_2$ genes was expressed in a yeast secretion system; 2) Agents that will activate T cells from the TCR/RAG mice were examined; both anti-CD3/anti-CD28 antibodies and a specific peptide were shown to stimulate proliferation; the peptide was used *in vivo* and optimal parameters for CTL-activation were determined. 3) An alternative bispecific agent, folate/anti-TCR antibody, for targeting high affinity folate receptors on tumor cells was further developed; these agents target tumors *in vitro* at very low doses (<0.1 nM). 4) Folate/antibody agents were used in TCR/RAG mice to evaluate parameters for efficient *in vivo* targeting of human tumors. This animal model will allow further testing of these and other bispecific agents.

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Dale M. King 10/29/97

PI - Signature Date

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INTRODUCTION
(Adapted in part from original proposal)

Successful treatment of breast cancer requires the identification of specific targets for the rational design of therapeutic agents. One such target, described several years ago, is the oncogene product erbB-2 (1-3). This protein is expressed on the surface of tumor cells in approximately 30% of women with the poorest prognosis for survival (4). The expression of erbB-2 does not appear to be correlated, either positively or negatively, with the estrogen receptor (5), although a recent report suggests that the erbB-2 signalling pathway targets the estrogen receptor resulting in estrogen-independent growth of tumor cells (6). However, it's expression is associated with an increase in the resistance of tumor cells to lysis by some natural immune mechanisms such as tumor necrosis factor and lymphokine activated killer cells (7,8).

A number of investigators have now begun to use antibodies specific for erbB-2 as possible therapeutic agents (9-16). The agents include immunotoxins, radioimmunoconjugates, and bispecific antibodies. The latter antibodies are intended to mediate effects by directing the lysis of tumor cells through cytotoxic effector cells. One of the predicted advantages of bispecific antibodies is that they should not have side effects associated with delivery of toxins or isotopes. Bispecific antibodies have in fact begun to be examined in several clinical trials, with some encouraging results.

Despite their promise and emergence into clinical trials, there are many questions that need to be addressed before optimal uses, with minimal side effects, of bispecific antibodies can be realized. For example, genetic engineering now provides a method for constructing smaller, potentially more stable, antibodies. Are these antibodies likely to be more effective than conventional intact antibodies? Will the natural resistance of erbB-2$^+$ tumors cells present an obstacle to successful bispecific antibody therapy? Can a system that uses a patients own immune cells be developed so that *ex vivo* activation of effector cells is not needed? This would obviously

allow the treatment of a much larger patient base than would be possible if effector cells must be cultured for every patient.

It would clearly be useful to have a system that could provide answers to these questions in order to design the most effective clinical trials of bispecific antibodies. There has been no animal model developed that can evaluate all of these issues using human breast cancer cells. The purpose of this project is twofold. First, to construct novel bispecific antibodies that can be expressed as a single-chain in *E. coli* (17-21). Second, to develop an *in vivo* animal model, using TCR transgenic and RAG-1 knockout mice (22-25). The animal model should allow the testing of the bispecific agents in comparison with other conventional bispecific antibodies. There are many potential therapeutic regimens that will need to be evaluated. To do so, an animal model that will not require introduction of human effector cells and that will most resemble the situation that will be encountered in the human disease will be developed. It is anticipated that these animals could also serve as models for immune modulating agents that are developed by other laboratories.

The four specific aims of the project are:

1) Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called $scFv_2$).

2) To use a simple screening method to search for agents that increase the sensitivity of erbB-2^+ breast cancer cell lines to lysis by CTL.

3) To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, $RAG^{-/-}$).

4) To test the *in vivo* effectiveness of various bispecific antibody regimens in the TCR/$RAG^{-/-}$ human xenograft system.

Since the submission of this grant application there have been a number of findings that have impacted the direction of the project. Perhaps chief among these is the basic understanding of how T cells are activated and inactivated (26-34). Understanding these processes are key to the rational development of bispecific antibodies that control and redirect the activity of T cells (35-39). It is especially relevant to this project that the activation of T cells is now known to be a complex process involving the triggering of multiple surface molecules (e.g. the T cell receptor/CD3 complex and the co-stimulatory molecule CD28). Given this complexity, it has become even more apparent to us that the successful application of bispecific antibodies in breast cancer therapy will require the use of the defined model system described in Specific Aim 4. Our success with this system, as summarized in this report, provides an opportunity to explore an additional direction (i.e. tumor-specific co-stimulation) that was only briefly alluded to at the time of the original grant application.

Accomplishments toward the specific aims of this project, in its third year, are provided in the body of this report. The Specific Aims are followed by a list of individual tasks and their status. The reviewer of last year's report raised a number of important issues and concerns regarding this project. The reviewer's comments, presented here in *italics*, are addressed in the appropriate sections below.

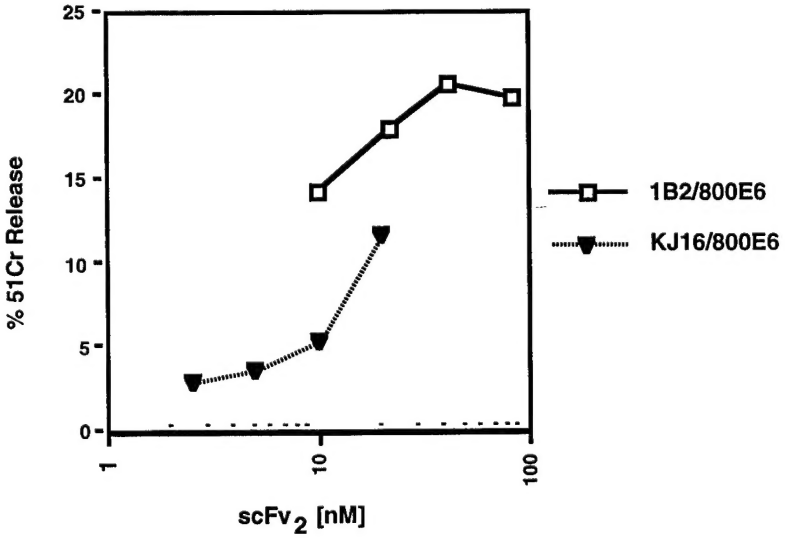
BODY

Specific Aim 1. Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called $scFv_2$).

In last year's report, the cloning and expression in *E. coli* of two bispecific $scFv_2$ were described. The scFv gene of anti-erbB-2 antibody 800E6 was cloned downstream of the anti-TCR antibodies 1B2 and KJ16 to produce two different $scFv_2$. 1B2 recognizes a clonotypic determinant on the TCR from CTL clone 2C (18) while KJ16 recognizes the Vβ8 chain of clone 2C (20). Both $scFv_2$ contained the 25 residue interchain linker 205 and a 10 residue c-*myc* tail for detection. These two antibodies were constructed in order to evaluate the effect of different antibody domains on folding of the $scFv_2$ and ultimately on their bispecific activity. Both $scFv_2$ were refolded from the denaturant by dilution and examined for binding of the TCR and erbB-2. As described previously, the KJ16/800E6 $scFv_2$ antibody (but not the 1B2 $scFv_2$) was found to bind specifically to the soluble TCR by ELISA and to the cell surface of CTL 2C by flow cytometry (data not shown). In addition, the KJ16/800E6 $scFv_2$ bound to erbB-2+ tumor cell line, BT474 as judged by flow cytometry. Finally, the 1B2/800E6 $scFv_2$ was shown to redirect lysis of the erbB-2^+ BT-474 tumor, but only at relatively high concentrations. Based on these findings, we hoped that the KJ16/800E6 $scFv_2$ would show more efficient redirected lysis than 1B2/800E6 $scFv_2$. This formed the basis of a new Task (9): Characterization of the KJ16/800E6 (anti-Vβ8/anti-erbB2) bispecific $scFv_2$).

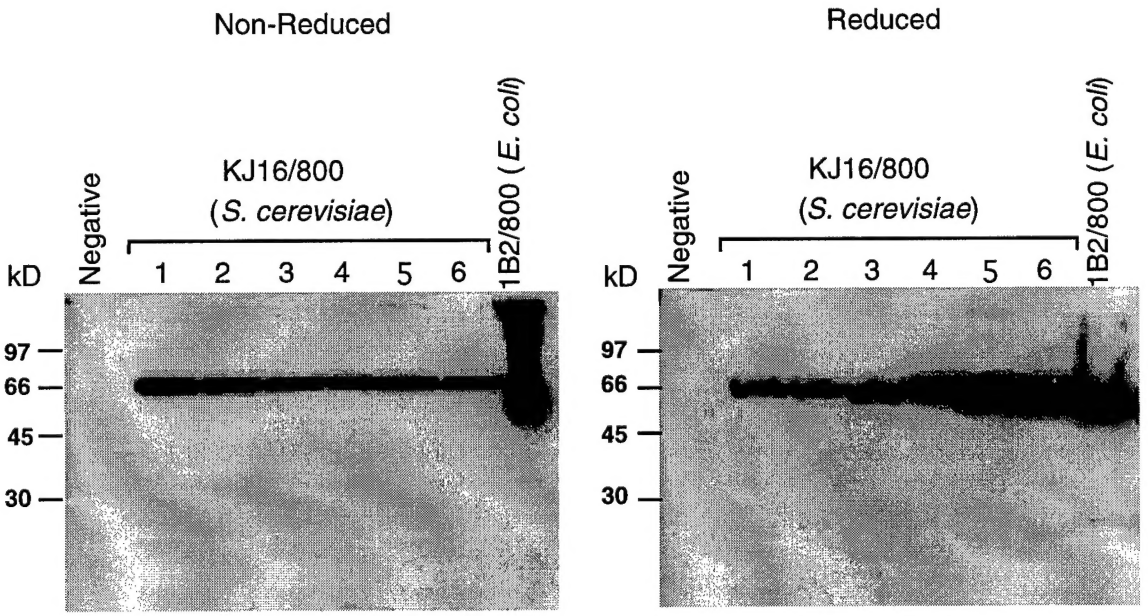
Figure 1 shows the extent of lysis specifically mediated by the KJ16/800E6 $scFv_2$ antibody. As shown for this KJ16/800E6 $scFv_2$ preparation (and another independently refolded preparation), there is not a significant difference between the ability of KJ16/800E6 $scFv_2$ and 1B2/800E6 $scFv_2$ to mediate lysis. These bispecific agents exhibit EC_{50} ~ 10 nM (EC_{50} = effective concentrations, equal to the concentration that yields 50% of the maximal lysis). This contrasts with the folate/antibody bispecific agents described below that use the same KJ16 scFv yet have EC_{50} ~ 0.1 nM (see Cho *et al, Bioconj. Chem.*, 1997 and Rund *et al.*, submitted, in Appendix). Thus, we believe that the activity of the *E. coli* derived $scFv_2$ bispecific preparations are not sufficient to merit use *in vivo*. Various alternate folding procedures were also evaluated with the KJ16/800E6 protein, but none yielded a higher fraction of properly folded protein (data not shown). As discussed further below, this does not mean that our work on bispecific antibodies has "come to a halt" (reviewer's words).

Figure 1. Cytotoxicity assay of BT-474 erbB-2^+ tumor cells with various concentrations of E. coli expressed $scFv_2$ and CTL clone 2C. Assays were performed at an E:T ratio of 10:1 (CTL 2C: BT474).



As indicated in last year's report, we also intended to explore the use of a yeast secretion system for $scFv_2$ proteins. (This approach was designated as: Task 10. Expression of $scFv_2$ (1B2/800E6 and KJ16/800E6) in a yeast secretion system). Our collaborator Dane Wittrup in the Department of Chemical Engineering here at the University of Illinois has shown that this system is capable of folding and secreting proteins with disulfide bond requirements, including scFv. KJ16/800 $scFv_2$ was cloned into the delta integration vector pITy2 NE0 (40). *Saccharomyces cerevisiae* was electroporated and intergrated $scFv_2$ transformants were selected on plates containing the antibiotic G418. Six colonies from this transformation were grown in culture and supernatants from each one were analyzed by Western blot with an anti-*c-myc* antibody (Figure 2). All six colonies showed a 65,000 dalton protein, under both reduced and non-reduced conditions. The protein migrated as a single, highly-resolved species, suggesting that it does not contain multiple different disulfide linked forms. The concentration of the protein was judged to be low (< 1 μg/ml), based on comparisons with the control $scFv_2$ isolated from *E. coli*. Nevertheless, this expression system may show some promise, if the material can be purified in larger quantities. Purification will be performed over the next year of the project, and the secreted $scFv_2$ will be assayed for binding to the TCR and erbB-2 and then assayed for targeting activity (see below for additional efforts with Fab x Fab antibodies).

Figure 2. Western analysis (anti-c-myc) of KJ16/800 $scFv_2$ secreted from *Saccharomyces cerevisiae*. Supernatants from six transformants (1-6) were run on an SDS-PAGE (reduced and non-reduced). As a negative control, supernatant from yeast not transformed with the $scFv_2$ was analyzed (negative).The $scFv_2$ 1B2/800 was expressed as inclusion bodies in *E. coli* under the control of a temperature inducible promoter. Inclusion bodies of 1B2/800 $scFv_2$ were isolated, denatured in guanidine-HCl and refolded in-vitro.



On page 2 of the review, the reviewer stated that *"there should be a growing concern about the feasibility of the goal of the project."* and that *"If the worst happened and the KJ16 turned out to have poor activity, the entire project would come to a halt and the task 4 would have to be repeated again"*. We do not share this view and take this opportunity to respond: First, the long-term goal of this project is to decide what bispecific agents will be the most useful clinically. A comparison of the various forms of such antibodies in an improved animal model remains an important goal. We proposed to produce and examine $scFv_2$ because they represent the smallest form yet described. However, we have always intended to keep an open mind about which of the

currently studied forms (conventional Fab x Fab, $scFv_2$, Fab-*Fos/Jun*-Fab or diabodies) might be optimal. In fact, companies such as Genentech and Protein Design Labs have taken a lead in producing stable, bispecific antibodies (41-44) and it is our belief that this aspect of the technology will not be the major hurdle. In contrast, companies tend to devote fewer resources toward improving animal models for comparing these products. Improved animal models can be extremely useful in that they guide the direction of expensive clinical trials only for the most promising agents.

Our study of the animal model has not come to a halt and, in fact, we have devoted considerable effort on this aspect of the project in the past year. As described below, we have continued to pursue targeting with bispecific agents (folate/antibody) that are further along than the $scFv_2$. We argue that these agents, combined with the TCR/RAG model, provide a rapid system to explore many parameters of T cell therapy. These parameters will be directly applicable to whatever form of the anti-erbB-2 bispecific antibodies we (or others) find most effective *in vitro*.

The reviewer also indicates that *"it is somewhat surprising that the PI has not proposed to make alterations to the linker between the two scFv fragments, as this would seem to entail about the same amount of effort. The results of these experiments will be interesting because they may point to future directions in the construction of bispecific Abs for redirected lysis"*. Actually, my lab explored the use of different intra-scFv linkers in a separate NIH funded project. The goal of that project was to link the 1B2 and KJ16 scFv in order to form a "high-affinity, bivalent agent". These attempts failed to identify better linkers that would allow the individual scFv domains to fold more efficiently. For this reason, we decided to focus on the yeast secretion system.

Because we continue to believe very strongly that bispecific agents against erbB-2 should be tested in animal models such as TCR/RAG mice, we will continue efforts to produce $scFv_2$ (in yeast) and Fab/Fab (see Specific Aim 4 below). In the next year of this award, the Fab/Fab and the purified $scFv_2$ from yeast strains will be examined *in vitro*. Our collaborator, Dane Wittrup has recently acquired the highest affinity scFv yet described (from Jim Marks) and also ~50 mg of the extracellular domain of erbB-2 (from Genentech). The Wittrup lab will be working out conditions for the expression of this anti-erbB-2 scFv in yeast. We will work with them on this project and on improving the expression levels of our $scFv_2$ genes.

Specific Aim 3. To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, $RAG^{-/-}$).

In last year's report, we described various properties of the TCR/RAG mice, that express a monoclonal population of $CD8^+$ T cells with the TCR from CTL clone 2C. These included the finding that TCR/RAG splenocytes could be activated *in vitro* with the superantigen Staphyloccocal enterotoxin B. In an effort to explore the optimal methods for activating these cells *in vivo*, two additional agents have now been tested *in vitro*. These include a 2C TCR-specific peptide SIYRYYGL (called SIYR here) and antibodies specific for CD3 and CD28. The synthetic peptide SIYR was discovered by random peptide screening for its ability 2C to sensitize K^b target cells for lysis by CTL 2C (45). Thus, we reasoned that smaller agents such as this peptide may be efficient in the *in vivo* activation of TCR/RAG T cells. In contrast, larger protein agents such as anti-CD3/CD28 antibodies are likely to have significant problems with penetration and *in vivo* clearance. On the other hand, antibodies have been used as the T cell activating agents of choice in many bispecific antibody targeting studies. In the future we imagine that either smaller anti-TCR/CD28 agents or generic peptides that stimulate a large fraction of the T cell repertoire might be used *in vivo*. For purposes of optimizing bispecific tumor targeting agents, many of our preliminary studies have been done with the SIYR peptide (see Specific Aim 4 below).

In vitro proliferation assays using spleen cells from TCR/RAG mice and various concentrations of SIYR (Figure 3A) and anti-CD3/anti-CD28 antibodies (Figure 3B) showed that both methods were capable of activation. In addition, activated cells were capable of recognizing and lysing P815, the normal L^d target cell for CTL 2C (data not shown). Given the significant activation observed with the peptide SIYR, various *in vivo* regimens were examined using i.p. injections of the peptide (Figure 4). Spleen cells were harvested from treated animals and assayed directly ex vivo using the P815 target cell. Spleen cells from TCR/RAG mice were not activated after injections of the mice with other peptides that are not recognized in the context of K^b (see Manning *et al. J. Immunol.* 1997, in Appendix). The results showed that 2.5 nmol peptide SIYR was capable of maximal activation of T cells (Figure 4B). Kinetic experiments showed that the peak activation occured one day after i.p. injection (Figure 4A and 4C). Finally, it was found that multiple injections were capable of restimulating CTL activity (Figure 4C). These results were used in the design of various *in vivo* targeting experiments with folate/antibody bispecific agents (see Specific Aim 4 below).

Figure 3. In vitro proliferation of TCR/RAG splenocytes induced by either SIYRYYGL peptide (A) or anti-CD3 and anti-CD28 antibodies (B). A. TCR/RAG$^{-/-}$ splenocytes (2 x 10^5 cells/well) were incubated for the indicated durations in the presence of various concentrations of SIYRYYGL peptide, and then assayed for proliferation by ^{3}H-thymidine incorporation during the last 18 hrs of incubation. **B.** TCR/RAG$^{-/-}$ splenocytes (2 x 10^5 cells/well) were incubated for 48 hrs with anti-CD3 antibody 2C11 (0.1 μg/ml), with or without anti-CD28 antibody 37.51 (5 μg/ml), and then assayed for proliferation by ^{3}H-thymidine incorporation.

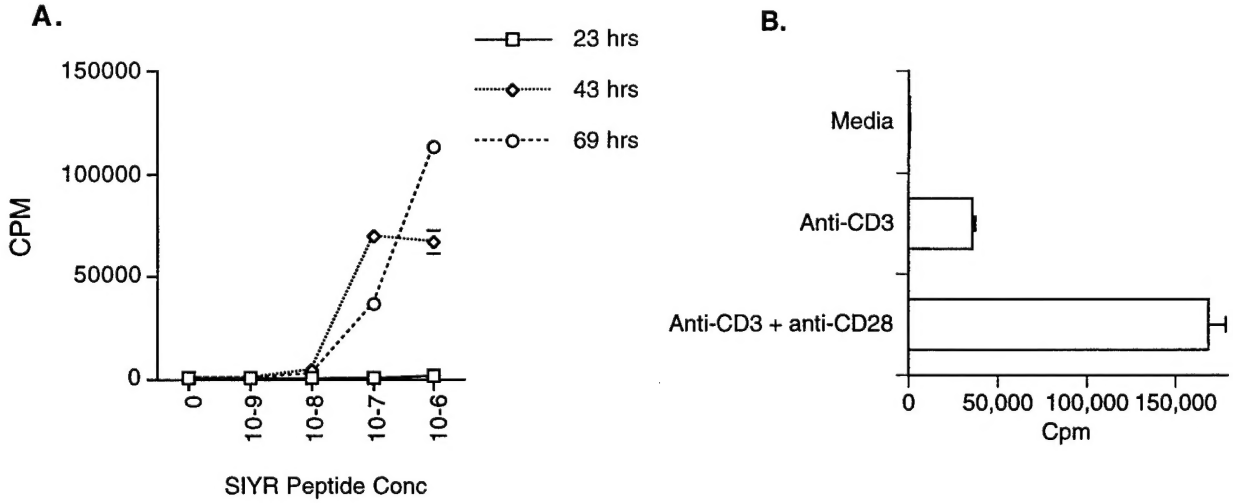
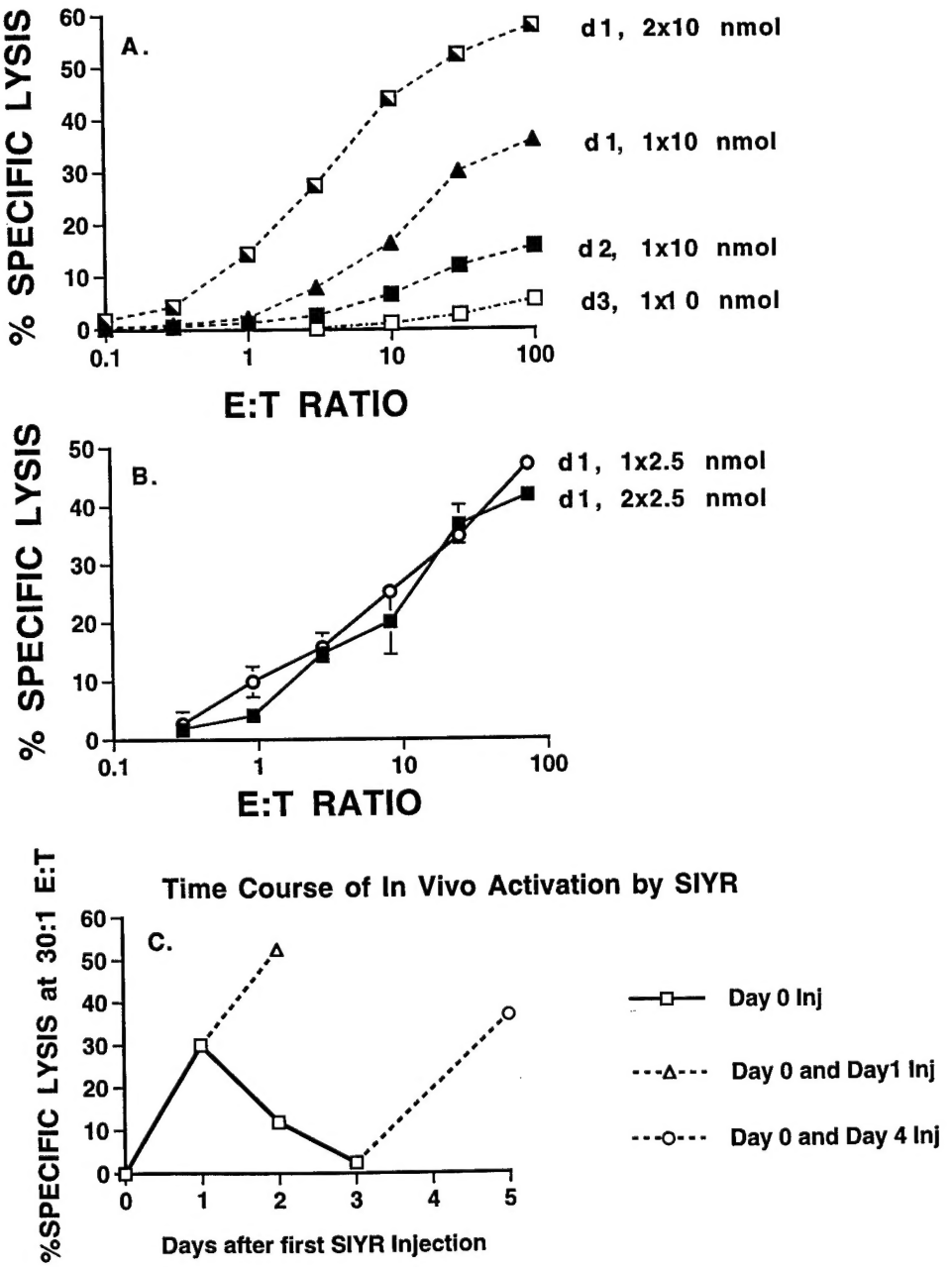


Figure 4. Ex vivo cytolytic activty of splenocytes following treatment of TCR/RAG mice with SIYRYYGL peptide. **(A)** Splenocytes were assayed 1, 2, or 3 days after a single 10 nmol i.p. injection of SIYRYYGL peptide (1 x 10 nmol); or 1 day after the second of two injections of 10 nmol peptide on consecutive days (2 x 10 nmol). Splenocytes were assayed at various E:T ratios against ^{51}Cr labeled P815 target cells. Standard error bars represent the variation of triplicate wells within the same assay. No cytolytic activity was observed with splenocytes from mice that did not receive peptide (data not shown). **(B)** With a 2.5 nmol dose of peptide, similar results were observed 1 day after a single injection (1 x 2.5 nmol) or 1 day after the second of two injections separated by 4 days (2 x 2.5 nmol). **(C)** Summary time course of cytolytic activity following treatments with SIYRYYGL peptide. Data from (A) and (B) at the 30:1 E:T are replotted as days versus cytolytic activity.



Specific Aim 4. To test the *in vivo* effectiveness of various bispecific antibody regimens in the TCR/RAG$^{-/-}$ human xenograft system.

Bispecific Antibody Preparations. In our original application, we proposed to compare various forms of bispecific antibodies, including conventional Fab x Fab. As briefly summarized above, our lab will soon be in the position to compare four different forms of the genetically engineered anti-erbB-2 $scFv_2$: 1) 1B2/800E6 $scFv_2$ refolded from *E. coli* inclusion bodies; 2) KJ16/800E6 $scFv_2$ refolded from *E. coli* inclusion bodies; 3) 1B2/800E6 $scFv_2$ secreted from yeast and 4) KJ16/800E6 $scFv_2$ secreted from yeast. We have now also succeeded in preparing the 1B2/800E6 Fab x Fab, using sulfhydryl chemistry (46).

800E6 and 1B2 $F(ab)'_2$ fragments were generated by digestion of intact IgG with ficin. The parent antibodies were incubated with ficin bound-agarose beads (Pierce) at 37^{0}C for 48 hours. The $F(ab)'_2$ fragments were run over a Protein A column to remove contaminating intact antibody. Purified $F(ab)'_2$ fragments (Figure 5, SDS-PAGE) from both antibodies were partially reduced to form F(ab)' fragments by incubation with 20mM cysteine for 30 minutes at 30^{0}C. The F(ab)' fragments were purified by HPLC over a G200 column (Figure 5, SDS-PAGE). The purified 800E6 F(ab)' fragments were reacted for one hour with 15mM ortho-phenyldimaleimide (o-PDM) in 35% dimethylformamide. The 800E6 F(ab)'-maleimide fragments were passed over a G25 column to remove excess o-PDM. The 1B2 F(ab)' and 800E6 F(ab)'-maleimide fraction were mixed and incubated overnight at 4^{0}C. The mixture was subjected to G200 HPLC chromatography to isolate the reoxidized 1B2/800E6 Fab x Fab as a 110 kDa species (Figure 5, SDS-PAGE). The Fab x Fab showed a major 55 kDa component which represents the covalently linked Fd from the 1B2 and 800E6 (Figure 5). This preparation binds to both the TCR and erbB-2 as monitored by flow cytometry using biotinylated, soluble TCR (Figure 6). The KJ16/800E6 will also be relatively simple to prepare, as we have now worked out conditions for the production of KJ16 $F(ab)'_2$ fragments using the enzyme ficin (data not shown).

Figure 5. SDS-PAGE profile of 1B2/800E6 IgG fragments. Proteins were stained with Coomasie Blue.

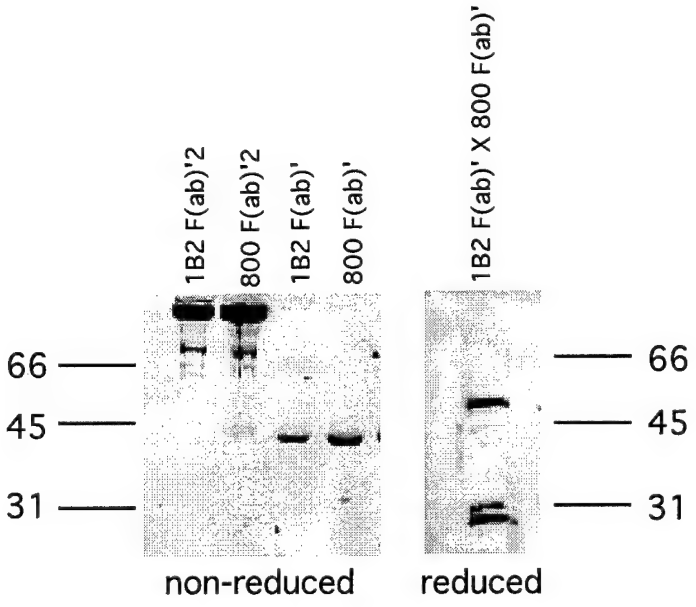
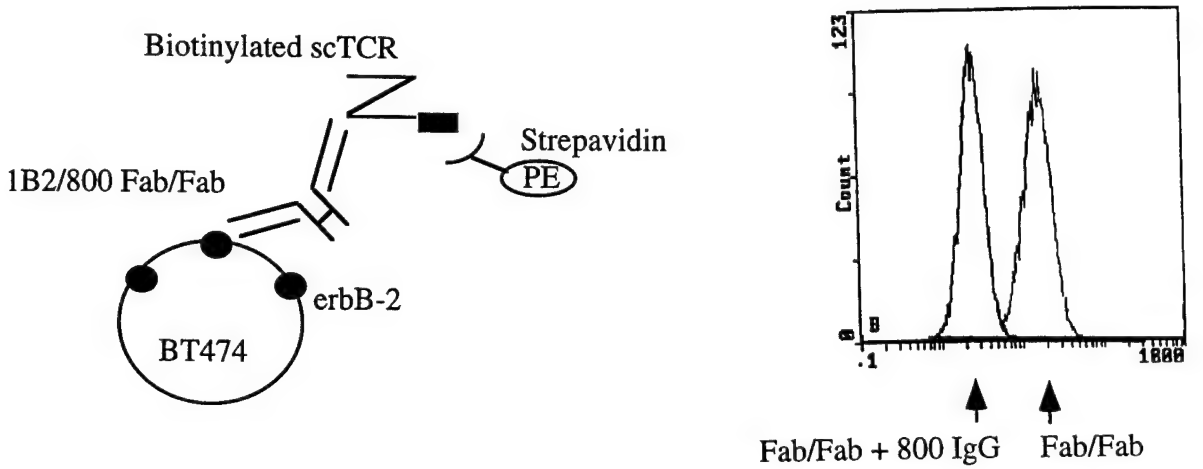


Figure 6. Flow cytometry of the purified 1B2/800E6 Fab x Fab, using BT-474 cells and a biotinylated TCR as detecting agent. BT-474 cells were incubated with the Fab x Fab preparation (~10 μg/ml). Various controls, including the addition of excess 800E6 IgG to compete with the Fab x Fab preparation were also examined. Note the increased fluorescence of the Fab x Fab incubated cells, indicating that both the erbB-2 and TCR binding domains are intact.



Thus, our goals in the next year are to compare six different bispecific antibodies for their ability to redirect the lysis of BT-474:

1B2/800E6 $scFv_2$ from *E. coli*
KJ16/800E6 $scFv_2$ from *E. coli*
1B2/800E6 $scFv_2$ secreted from yeast
KJ16/800E6 $scFv_2$ secreted from yeast
1B2/800E6 Fab x Fab
KJ16/800E6 Fab x Fab

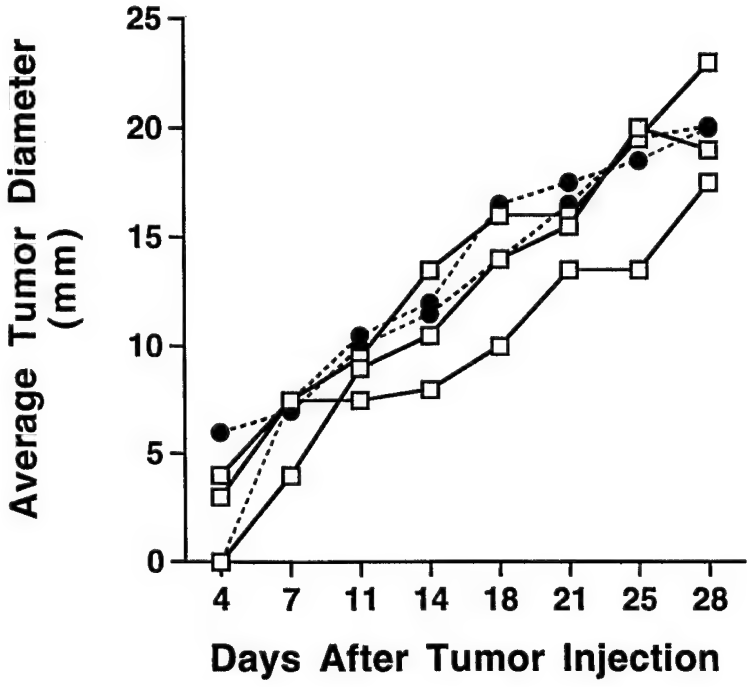
Based on the results shown in Figure 1, we do not expect the *E coli* derived forms to have optimal activity. Our intentions are to see if in fact the yeast secreted $scFv_2$ activity approaches that of the Fab x Fab. If so, all of these proteins would be compared *in vivo*. If these $scFv_2$ proteins are not as active (e.g. < 10 times the activity of Fab x Fab), then we have two choices. First, to explore other expression systems with these scFv, such as the Fos/Jun system of Protein Design Laboratories. Our lab has already begun to clone Fos/Jun domains onto the scFv-KJ16 for an NIH-sponsored project. Alternatively, we could align ourselves with a company that currently has anti-human CD3 bispecific conjugates (such as Protein Design Labs) and put all of our efforts into the development of the next generation animal model. This would involve crossing the human CD3ε transgenic mice with our TCR/RAG mice. This project is one that we intend to pursue in any case, as it will allow the direct testing of human clinical agents in the mouse.

Effectiveness of Bispecific Folate/Antibody Conjugates in the TCR/RAG Model. The reviewer has commented that *"If the worst happened and the KJ16 turned out to have poor activity, the entire project would come to a halt"*. If the only bispecific agent that we had in development were the $scFv_2$, then this arguably may have been a valid conclusion, from the standpoint of TCR/RAG experiments. However, as described in our earlier reports, our lab has been simultaneously developing a new class of bispecific agents that have the same purpose as the anti-erbB-2 conjugates (i.e. to target tumor cells for lysis by T cells). These bispecific agents are

conjugates of the small molecule folate (440 daltons) and an anti-TCR antibody (19, 21). A high-affinity folate receptor (FR) has been shown to be expressed on most ovarian and many breast cancer cells (47). In fact, the first generation of anti-FR/anti-CD3 antibodies are in clinical trials for ovarian cancer (48). We have shown that the folate/antibody agents have potent tumor targeting activity, either as folate/IgG, folate/Fab, or folate/scFv conjugates. Thus, the folate conjugates provide a useful system to begin to evaluate various aspects of the TCR/RAG model. It is important to realize that these experiments are directly applicable to this project, as the findings will impact how erbB-2-specific antibodies can be used to optimally recruit and redirect T cells.

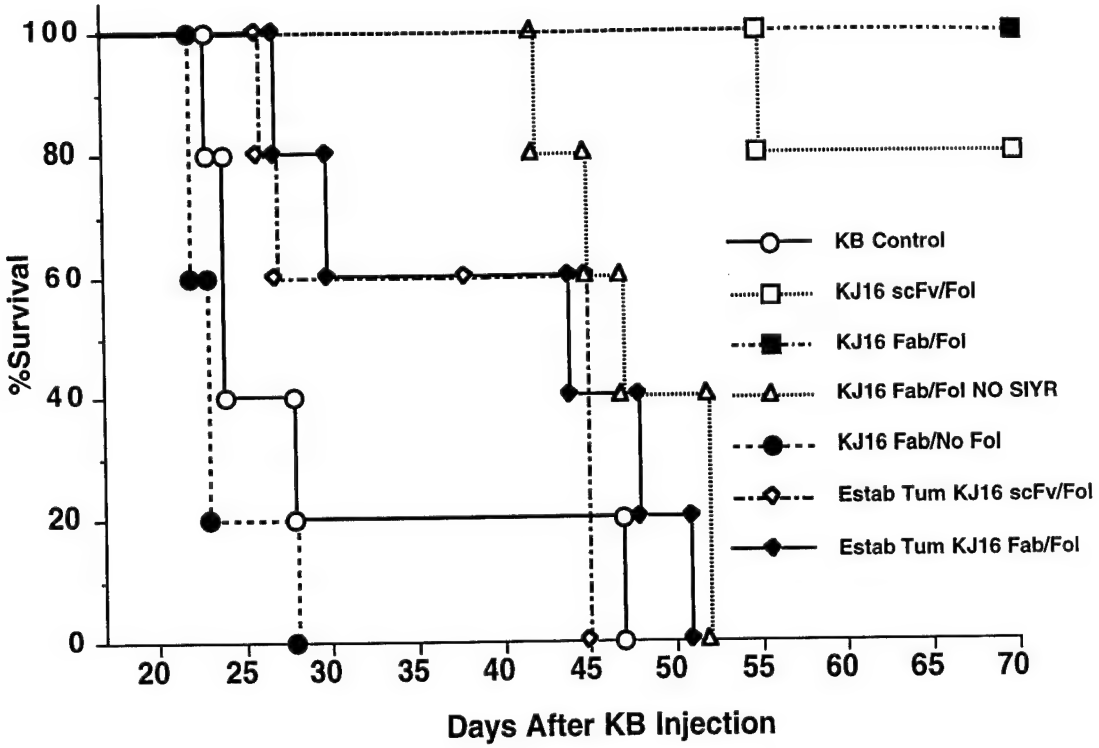
A complete presentation of our results with folate/KJ16 antibody conjugates and human FR^+ tumors in TCR/RAG mice are provided in the attached manuscripts (Cho *et al. Bioconj.Chem.*, 1997 and Rund *et al.*, submitted to *Cancer Research*). Several key experiments involving the human FR^+ tumor KB are summarized here. As shown in the previous report for several erbB-2^+ tumors, KB grew in TCR/RAG mice, even when T cells had been activated *in vivo* with SIYR peptide (Figure 7).

Figure 7. Tumor growth curves of KB xenografts in TCR/RAG mice treated with SIYRYYGL peptide. Mice treated one day previously with 2.5 nmol SIYRYYGL peptide (□, n=3) or without SIYRYYGL peptide (●, n=2), were injected s.c. with KB cells ($1x10^7$ in 200µl). Vernier calipers were used to take 2 perpendicular tumor measurements twice weekly. Measurements were averaged to determine mean tumor diameter.



Various treatment groups were examined in order to evaluate several issues regarding bispecific antibody use in this model: 1) folate/antibody vs antibody alone to evaluate specificity of the treatments; 2) comparison of folate/Fab vs folate/scFv; 2) effect of T cell activation (with SIYR) prior to bispecific antibody treatment; 3) effects of co-injecting antibodies and tumor vs treatment after tumor has been established. The most relevant results regarding these issues are shown in Figure 8.

Figure 8. Effects of folate/antibody conjugates on the survival of TCR/RAG mice with peritoneal KB tumors. KB cells ($5x10^6$) mixed with PBS (○), 10 μg KJ16 Fab fragments (●), 10μg folate/KJ16 Fab fragments (□), or 10μg folate/scFv (□) were injected i.p. into TCR/RAG mice (n = 5 per treatment) as a single cell suspension in 1 ml. Antibodies were administered twice, mixed with the cells and 4 days later. For the established groups, antibodies wee added on days 2 and 6 after tumor injection (established folate/Fab fragments ; established folate/scFv ◆). In all except the NO SIYRYYGL treatment group (△), 2.5 nmol of SIYRYYGL peptide was administered i.p. 24 hr. prior to each antibody injection. The NO SIYRYYGL treatment group (△) received 10μg folate/KJ16 Fab fragments mixed with KB cells as in experimental group (□). Mice were monitored daily for signs of illness and were euthanized as they became moribund. (see Rund *et al.* in Appendix for additional details).



From these results, we have concluded that prior T cell activation and access of the bispecific agents to the tumor (as is optimally achieved in the co-injection experiment) are both critical to the success of therapy. Access of the bispecific agent could either be achieved by binding of the agent directly to the tumor (as is the main consideration in most forms of antibody therapy) or alternatively by infiltration of T cells that carry the bound conjugates. To the best of our knowledge, there is little information on which avenue of delivery might prove most successful (or in fact which is operative in the many studies performed with bispecific antibodies). Our own view is that improvements will be best directed at increasing the extent of T cell infiltration into tumors. T cells are known to extravasate into tumors under conditions that are not optimal for efficient antibody delivery. Thus, we propose to explore additional strategies for oprtimizing T cell activity and sustaining it at the site of the tumor. This will include the use of anti-CD28-directed agents, which are now well known to yield maximal T cell stimulation (26-34). In addition, we have recently collaborated with Jeff Bluestone's lab on the engineering of their anti-CTLA-4 antibody 4F10 (29). Fab fragments of this antibody are effective at increasing T cell activity, presumably by blocking the normal CTLA-4 signals that lead to T cell inactivation. Our future efforts will include using combinations of folate/anti-CD28 Fab fragments and anti-CTLA-4 Fab fragments in the TCR/RAG model.

CONCLUSIONS

Work Completed. Tasks shown below with an * have now been completed and significant progress was made on tasks indicated with an **:

***Task 1,** Cloning, expression, and testing of erbB-2 single-chain antibody, months 1-12.

***Task 2,** Screening of tumor cell lines for susceptibility to CTL-mediated lysis using the anti-fluorescein bispecific antibody, months 1-12.

***Task 3,** Breeding of transgenic TCR/RAG$^{-/-}$ mice and testing of peripheral blood T cells for reactivity with 1B2 antibodies, months 1-20. It is anticipated that approximately 175 mice will be produced by the end of this period.

***Task 4,** Cloning, expression, and *in vitro* testing of bispecific single-chain scFv$_2$ antibody (1B2/erbB-2), months 13-24.

***Task 5,** Screening of tumor cell lines for increased susceptibility to CTL-mediated lysis when tumor cells are treated with: anti-erbB-2 antibodies, IFN-γ, TNF-α, estrogen, tamoxifen, months 13-30.

***Task 6,** Transplantation of various erbB-2^{+} tumor cell lines into TCR/RAG$^{-/-}$ mice and evaluation of tumor incidence, months 16-36. It is anticipated that approximately 15 mice per month will be used.

****Task 7,** Purification and *in vitro* testing of bispecific Fab$_2$ antibody (1B2/erbB-2), months 30-48.

****Task 8,** *In vivo* testing of bispecific antibodies in TCR/RAG$^{-/-}$ mice that have received human tumor transplants, months 30-48. It is anticipated that approximately 15 mice per month will be used. This task now includes a continuation of our efforts with the human KB tumor model and folate/antibody conjugates.

***Task 9.** Characterization of the KJ16/800E6 (anti-Vβ8/anti-erbB2) bispecific scFv$_2$, months 30-48.

****Task 10.** Expression of scFv$_2$ (1B2/800E6 and KJ16/800E6) in a yeast secretion system, months 30-48.

****Task 11.** Comparison of *in vivo* activation strategies in TCR/RAG$^{-/-}$ mice, months 30-48. This task includes our studies with anti-CD28 bispecific antibodies and soluble forms of the anti-CTLA-4 Fab fragments to sustain the activity of T cells.

Publications of the Principle Investigator During the Past Year (*Directly related to this project; provided in Appendix):

Schodin, B.A., T. J. Tsomides, and D. M. Kranz (1996) Correlation Between the Number of T Cell Receptors Required for T Cell Activation and TCR-Ligand Affinity. *Immunity* 5: 137-146.

Schodin, B. A., C. J. Schlueter, and D. M. Kranz (1996) Binding Properties and Solubility of Single-Chain T Cell Receptors Expressed in*E. coli. Mol. Immunol.* 33: 819-829.

Schlueter, C. J., T. C. Manning, B. A. Schodin, and D. M. Kranz (1996) A Residue in the Center of Peptide QL9 Affects Binding to Both L^d and the T Cell Receptor. *J. Immunol.* 157: 4478-4485.

*Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123.

*Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1997) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. *Bioconj. Chem.* 8:338-346.

*Kranz, D. M., T. C. Manning, L. A. Rund, B. K. Cho, and E. J. Roy (1997) Targeting Tumor Cells with Bispecific Antibodies and T Cells. *J. Control. Release.* In Press.

*Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in $CD8^+$ TCR Transgenic/$RAG^{-/-}$ Mice. *J. Immunol.* In Press.

Kieke, M. C., B.K. Cho, E.T. Boder, D.M. Kranz, and K.D. Wittrup (1997) Isolation of Anti-T Cell Receptor scFv Mutants by Yeast Surface Display. *Protein Engineering.* In Press.

Cho, B. K., M. C. Kieke, E. T. Boder, K. D. Wittrup, and D. M. Kranz (1997) A Yeast Surface Display System for the Discovery of Ligands that Trigger T Cell Activation. Submitted.

*Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick (1997) Targeting T Cells Against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. Submitted.

*Rund, L. A., B. K. Cho, T. C. Manning, E. J. Roy, and D. M. Kranz (1997) A Transgenic Model to Test Bispecific Antibody Targeting of Endogenous T Cells Against Human Tumors. Submitted.

In addition, this project was selected for a platform presentation at the DOD *Era of Hope* meeting (Oct 31-Nov 4, 1997).

Future Work:

During the next year, we intend to work on the tasks indicated above by an **. These will specifically include:

- Purification of $scFv_2$ (1B2/800E6 and KJ16/800E6) from yeast
- Purification of Fab x Fab (KJ16 x 800E6)
- Testing of these and other forms described in this report in vitro
- Preparation of folate/anti-CD28 Fab conjugates
- Preparation of anti-CTLA-4 Fab fragments
- Testing of the latter two agents in the TCR/RAG model, using KB tumor cells.

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Appendix

Six papers/manuscripts that involve this project are provided in the Appendix:

*Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1997) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* 32: 111-123.

*Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1997) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. *Bioconj. Chem.* 8:338-346.

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*Manning, T.M., L.A. Rund, M. Gruber, F. Fallarino, T. F. Gajewski, and D. M. Kranz (1997). Antigen Recognition and Allogeneic Tumor Rejection in $CD8^+$ TCR Transgenic/$RAG^{-/-}$ Mice. *J. Immunol.* In Press.

*Roy, E. J., B. K. Cho, L. A. Rund, T. A. Patrick (1997) Targeting T Cells Against Brain Tumors with a Bispecific Ligand-Antibody Conjugate. Submitted.

*Rund, L. A., B. K. Cho, T. C. Manning, E. J. Roy, and D. M. Kranz (1997) A Transgenic Model to Test Bispecific Antibody Targeting of Endogenous T Cells Against Human Tumors. Submitted.

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Laboratory Investigation

Folate receptors as potential therapeutic targets in choroid plexus tumors of SV40 transgenic mice

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Key words: brain tumor, choroid plexus carcinoma, ependymoma, SV40 T antigen, folate binding protein, SV11 mice

Summary

A high affinity folate receptor is expressed in some human cancers, including choroid plexus tumors and ependymomas, and has been suggested as a target for therapeutics. In this report, the expression of folate receptors in an SV40 large T antigen transgenic mouse (SV11) was investigated. SV11 mice develop choroid plexus tumors, a property that may be related to the observation that SV40 has been isolated from human choroid plexus tumors and ependymomas. We report that SV11 choroid plexus tumors contain a high affinity folate receptor (K_D of 1 nM), detectable by ^{125}I-folate autoradiography and immunohistochemistry. Western blot analysis indicated an apparent molecular weight of 38 kDa. RT-PCR revealed the presence of transcripts for both alpha and beta isoforms of the folate receptor. Brain parenchyma has undetectable folate receptor, but normal choroid plexus has substantial levels (as does human choroid plexus). The folate receptors of the tumor are accessible from the bloodstream whereas those of the normal choroid plexus are not. Thus SV11 transgenic mice should be useful for evaluating therapeutic targeting of high affinity folate receptors, both for efficacy of specific agents and possible side effects.

Introduction

The choroid plexus is a specialized region of ependyma, the epithelial lining of the brain ventricles. The incidence of choroid plexus tumors and ependymomas is disproportionately high in young children: 40–70% of each type of tumor is diagnosed in children under two years of age [1, 2]. Pediatric ependymomas and choroid plexus tumors may have an unusual viral etiology. The genomic sequence of the SV40 viral large T antigen (Tag) was detected by PCR in nearly all pediatric ependymomas and in half of choroid plexus tumors examined [3]. The large T antigen was detectable by immunohistochemistry in these tumors. In contrast, other tumor types did not have detectable viral DNA or immunoreactivity. Furthermore, the SV40 virus has subsequently been recovered from the same tumor samples [4].

Mice that are transgenic with SV40 develop ependymal and choroid plexus tumors [5, 6]. One particular line, SV11, develops tumors exclusively in the choroid plexus, and the mice become moribund at 104 ± 12 days [7]. The mice are transgenic for the early control region and early genes of the SV40 genome encoding the large T antigen (Tag). Tag is a nuclear protein capable of binding both p53 and pRB; Tag binding of pRB is necessary for Tag-induced tumorigenesis [8]. Tag is expressed in some choroid plexus cells soon after birth and in most

cells when tumors progress to Grade III and IV at approximately 80 days of age [7]. The SV11 transgenic mice are potentially a valuable animal model for human brain tumors, because the tumors arise endogenously in the normal physiological context and the etiology mimicks that of a subset of pediatric brain tumors.

A number of therapeutic strategies require the identification of surface molecules on tumors that can serve as targets for effector mechanisms such as toxins and immune cells. The surface marker need not be unique to the tumor, but should be expressed at higher levels on tumor cells than on normal cells. One candidate surface antigen is the high affinity folate receptor (FR), a GPI-linked glycosylated monomer of approximately 38 kDa originally isolated by monoclonal antibodies to ovarian carcinomas [9–12].

FRs mediate via potocytosis the uptake of various serum folates, that serve as carbon donors for purine and thymidine synthesis. Distinct FR isoforms that exhibit greater than 70% homology have been identified in humans (FR-α, FR-β, and FR-γ) and in mice (FR-α and FR-β) [13–18]. The affinity of FRs for folate is of the order of 1 nM K_D. The exact role of the high affinity folate receptor remains unclear. A low affinity reduced folate carrier system ($K_D \approx 1$–100 μM) [19, 20] is capable of providing for the folate requirements of normal cells. Interestingly, while FR is not necessary to meet the metabolic requirements of tumor cells *in vitro*, neoplastic cells transfected with FR are capable of increased folate acquisition and enhanced cell growth [21, 22].

FR-α has been found to be over-expressed in most human ovarian carcinomas and approximately 30% of other carcinoma varieties including many mammary adenocarcinomas [9, 23, 24]. FR-β is highly expressed in some mammary carcinomas and a variety of nonepithelial tumors [24]. The isotype of FR in human choroid plexus tumors has not been characterized.

Folate receptors have been used as a possible therapeutic target in various ways. Anti-folate drugs such as 5, 10-dideazatetrahydrofolic acid, CB3717, or ICI-198,583 are believed to mediate anti-tumor activity after accumulation by FRs [25]. Other chemotherapeutics and liposomes have been specifically delivered to FR-bearing cells via chemical conjugation to folic acid [26, 27]. Bispecific antibodies that contain anti-FR antibodies linked to anti-T cell receptor antibodies have been used to target T cells to FR-positive tumor cells and are currently in clinical trials for ovarian carcinomas [28, 29]. Conjugates of folic acid and anti-T cell receptor antibodies are effective at targeting FR-positive tumors with cytotoxic T cells [30].

In this study, we analyzed the anatomical and molecular characteristics of FRs expressed by choroid plexus tumors and normal choroid plexus epithelium in SV11 transgenic mice.

Materials and methods

Cell lines

The following DBA/2-derived tumor cell lines generously provided by Kevin Brigle were used to characterize reagents used in the analysis of SV11 FRs. Cells were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 units/l penicillin, and 100 μg/ml streptomycin: Mel, murine erythroleukemia cell; Mel La, a subline of Mel selected on low folate; L1210, a murine leukemia line; LL3, a subline of L1210 selected on low 5-formyltetrahydrofolate; and F2-MTX^{r}A, a MTX resistant L1210 subline selected on low folate. Mel La and LL3 express primarily FR-α; F2-MTXrA expresses only the FR-β isoform.

Transgenic mice

SV11 males heterozygous for the SV40 early control region and early genes were mated to C57BL/6J females (Jackson Laboratories, Bar Harbor, ME). Progeny were screened at one month of age for the Tag gene by PCR. A 1 cm section of tail was digested in 600 μl 0.5 M Tris pH 8.0, 0.1 M EDTA, 0.1 M NaCl, 1% SDS with 100 μg of proteinase K overnight at 55° C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. DNA was resuspended in TE (10 mM Tris pH 8.0, 1 mM

EDTA) and used in PCR with primer pairs specific to regions of both Tag and transthyretin (an endogenous sequence used as a control).

Western blot analysis

Membranes were prepared by homogenizing approximately 50 mg of tissue in 5 mM Tris pH 7.4, 5 mM sucrose, 100 μg/ml phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin. Homogenates were centrifuged at 1,500 × g to remove nuclei and supernatants were brought to 10 mM $CaCl_2$ followed by centrifugation at 30,000 × g for 30 min at 4° C. Membrane pellets were solubilized in extraction buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) on ice for 20 min and centrifuged at 30,000 × g to remove insoluble material. Approximately 25 μg membrane protein from tumor and 40 μg membrane protein from cortex were electrophoresed through 10% polyacrylamide gels in SDS under non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes for 1 hr. Membranes were blocked with TBST (10 mM Tris base, 0.15 M NaCl, 0.05% (v/v) Tween 20) with 1% BSA for 1 hr. FRs were detected with rabbit anti-FR antiserum (generously provided by Kevin Brigle) followed by biotinylated goat anti-rabbit and ABC (Vector Elite, Burlingame, CA). The chromagen was diaminobenzidine enhanced with nickel-cobalt (DAB, Pierce, Rockford, IL).

Autoradiographic analysis of folate binding to tissue sections

Brains from tumor bearing mice were frozen in mounting medium and sections cut at 10 μm. Sections were incubated in 0.2 M sodium acetate buffer pH 4.5 to remove unlabeled folate, washed in 50 mM Tris 150 mM NaCl buffer, pH 7.6, and incubated with 100 μl of one of seven concentrations from 0.01 nM to 2.6 nM of ^{125}I-labeled folic acid (Amersham, S.A. 2000 Ci/mmol) at room temperature in a humid chamber for 30 min. Control slides were incubated with isotope plus 100-fold excess unlabeled folic acid. Non-specific binding was less than 30% of total binding at the highest concentration. Slides were washed 3 times in Tris saline buffer, and dried in a dessicator at 4° C. After drying, slides were dipped in Kodak NTB-3 emulsion at 42° C, air dried, and stored in a dessicated box until developed 24 hrs later. Autoradiograms were analyzed using the program NIH Image to quantify silver grains.

Immunohistochemistry

Immunohistochemistry was performed using the same polyclonal rabbit antibody against the FR used in the Western blots. Mice were anesthetized and perfused with saline followed by AZF (acetic acid, zinc chloride, 10% formalin, Newcomer Supply, Middleton, WI). Similar immunoreactivity was obtained with perfusion fixation with AZF, immersion fixation with AZF, immersion fixation with AMeX (acetone, methyl benzoate, xylene), or with cryostat sections of unfixed tissue (data not shown). Sections were blocked with 5% normal goat serum and endogenous peroxidase activity quenched with 3% H_2O_2 in methanol. Six primary antibody dilutions of polyclonal anti-FR antiserum ranged from 1 : 1,000 to 1 : 50,000 (5 μg/ml – 0.1 μg/ml) in Biomeda antibody buffer. Secondary antibody (1 : 1,000) was biotinylated goat anti-rabbit IgG (Vector), followed by avidin-biotin peroxidase complex (ABC, Vector). The chromagen was metal enhanced DAB (Pierce).

RNA isolation

Total RNA was isolated from approximately 100 mg tumor tissue or 10^8 cultured cells by solubilization in guanidine hydrochloride and differential centrifugation through a CsCl gradient [31]. RNA pellets were solubilized in H_2O, ethanol precipitated, and re-solubilized in H_2O. Concentrations were calculated from A_{260} readings.

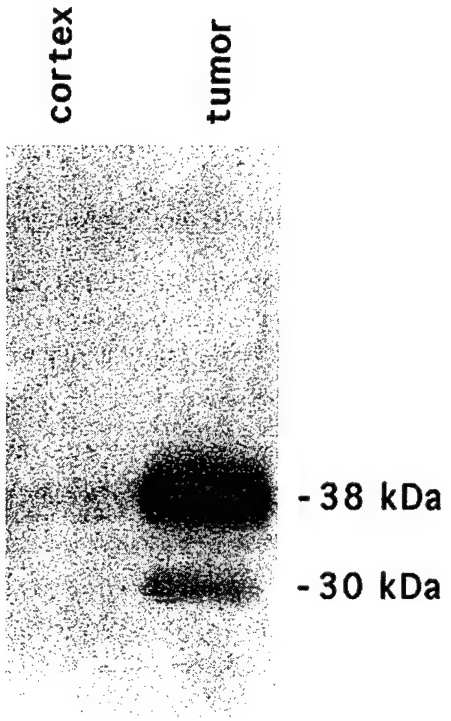


Figure 1. Western blot analysis of membrane proteins. Samples of choroid plexus tumor and surrounding brain cortex were homogenized and membranes isolated by differential centrifugation; proteins were detergent solubilized and electrophoresed through 10% polyacrylamide gels (25 µg of tumor protein and 40 µg of cortex protein). After transfer to nitrocellulose membranes, proteins were detected with a polyclonal antiserum against the mouse folate receptor.

Northern blot analysis

Forty µg total RNA was denatured in 0.5 X formaldehyde gel-running buffer (1X : 0.02 M 3-(N-morpholino)propanesulfonic acid pH 7.0, 8 mM sodium acetate, 1 mM EDTA), 2.2 M formaldehyde, and 50% (v/v) deionized formamide and the sample was electrophoresed through a 1.5% agarose gel containing 0.66 M formaldehyde and 1 X formaldehyde gel-running buffer. Molecular weight marker lanes were stained separately in 5 µg/ml ethidium bromide, 0.1 M ammonium acetate for 15 minutes and destained in 0.1 M ammonium acetate for two hrs. RNA was transferred to nylon membranes by capillary action in 20 X SSC (1 X SSC: 0.15 M NaCl, 0.015 M Na_3 citrate) for a minimum of 16 hrs. Membranes were washed with 2 X SSC and dried prior to baking at 80° C for 2 hrs.

Probes for FR-α and FR-β were made from isolated cDNAs of each (provided by Kevin Brigle). DNA was labeled using a random primer kit (Gibco BRL, Gaithersburg, MD) and [α-^{32}P]-dATP (ICN, Costa Mesa, CA) according to the manufacturer's instructions. Membranes were pre-hybridized for 1 hr at 65° C in 0.52 M sodium phosphate (pH 7.2), 15 mM EDTA, 0.25 M NaCl, 6.4% SDS, and 0.01% BSA with 4 µl/ml denatured sonicated salmon sperm DNA. 20 µl heat-denatured [α-^{32}P]-labeled probe and an additional 4 µg/ml denatured sonicated salmon sperm were added to the hybridization buffer and allowed to hybridize for a minimum of 12 hrs at 65° C. Membranes were washed successively in 2 X SSC, 0.1% SDS at room temperature, 2 X SSC, 0.1% SDS at 65° C, and 0.2 X SSC, 0.1% SDS at 65° C. The sizes of the major transcripts were calculated by a linear regression plot derived from the marker lane (0.16–1.77 Kb RNA Ladder, Gibco BRL).

PCR of FR-α and FR-β cDNA

Plasmid containing cDNAs for FR-α or FR-β was serially diluted by ten-fold increments (5 ng/µl – 500 fg/µl). 1 µl aliquots of each dilution were added to 100 µl of 1 X PCR buffer (50 mM KCl, 10 mM Tris pH 8.4, 1.5 mM $MgCl_2$, 50 mM dNTPs) containing primer pairs (200 nM each) specific to either FR-α or FR-β and 1 unit of Taq polymerase (Fisher). Primer pairs for the FRs amplified the entire coding regions (765 bp for FR-α and 753 bp for FR-β) of each respective cDNA. Each respective set of FR primer pairs flank introns such that amplification of genomic DNA is readily distinguished from cDNA. Sequences of primer pairs were as follows:

FR-α5′ ATGGCTCACCTGATGACTGTGCAG
FR-α3′ GCTGATCACCCAGAGCAGCACTAA
FR-β5′ ATGGCCTGGAAACAGACACCACTC
FR-β3′ GCCAGGGAGCCATAATGACAGCAC

PCR was carried out a total of 35 cycles with a denaturing temperature of 94° C and elongation tem-

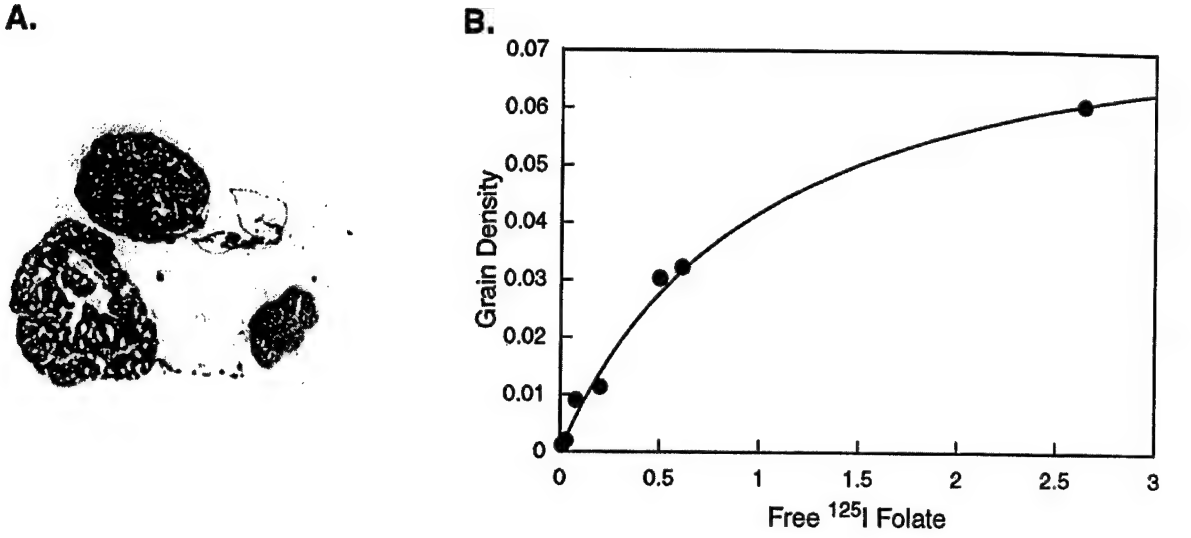


Figure 2. Autoradiographic detection of folate receptor binding using ^{125}I-folate on tissue sections. Cryostat sections of unfixed brain and tumor were incubated with varying concentrations of labeled folate (0.01 nM to 2.6 nM) for 30 min. Slides were dipped in photographic emulsion and exposed for 24 hrs. Sections were counterstained with hematoxylin. *A*. Labeling at 2.6 nM ^{125}I-folate. *B*. For each concentration of labeled folate, silver grains were counted using the computer program NIH Image. Nonlinear regression analysis of the binding isotherm yielded a K_D of 1 nM.

perature of 72° C for each set of primers. Annealing temperature for FR primer pairs was 57° C.

RT-PCR

5 µg of total RNA isolated from cell lines or tumor was heat denatured and added to 1 X cDNA reaction buffer (50 mM KCl, 10 mM Tris pH 8.4, 5 mM DTT, 2.5 mM $MgCl_2$, 0.5 mM dNTPs) with 0.3 µl RNAsin (Promega, Madison, WI), 5 mM oligo-dT, and 45 units AMV reverse transcriptase (Seikagaku, Japan) in a total reaction volume of 20 µl. Reverse transcription reactions were carried out at 42° C for 1 hr. 1 µl of the resulting cDNA reaction mixture was aliquoted into PCR buffer containing either FR-α or FR-β primers and amplified as described.

For RT-PCR analysis of FR isoform expression in tumor and choroid, 0.5 µg of total RNA isolated from normal choroid plexus or tumor was examined as described above to evaluate relative FR isoform expression between tissue samples. Normal choroid plexus was dissected from C57BL/J6 mice 2 hrs after intravenous injection of 0.1 ml 5% (w/v) Evan's Blue dye to facilitate visualization of the choroid plexus. Two-fold dilutions of cDNA reaction mixtures (ranging from 2 µl – 1/256 µl) were used in PCR. β-actin primers, amplifying a 193 bp fragment of β-actin, served as a control for the level of intact mRNA in different samples. Similar to FR primers, β-actin primers flanked at least one intron. Sequences of β-actin primers were as follows:

β-actin 5′ TGCTCTAGACTTCGAGCAGGAG
β-actin 3′ CATGATGGAATTGAATGTAGTT

Southern blot analysis of RT-PCR products

Ten µl of each PCR reaction mixture was electrophoresed on a 1.5% agarose gel containing 5 µg/l ethidium bromide. Gels were washed in 0.2 M HCl for 30 min followed by 0.2 M NaOH, 0.6 M NaCl for 30 min, and finally 0.5 M Tris pH 7.5, 1.5 M NaCl for 30 min. DNA was transferred by capillary action to Genescreen nylon membranes in 20 X SSC for a minimum of 12 hrs. Membranes were pre-treated

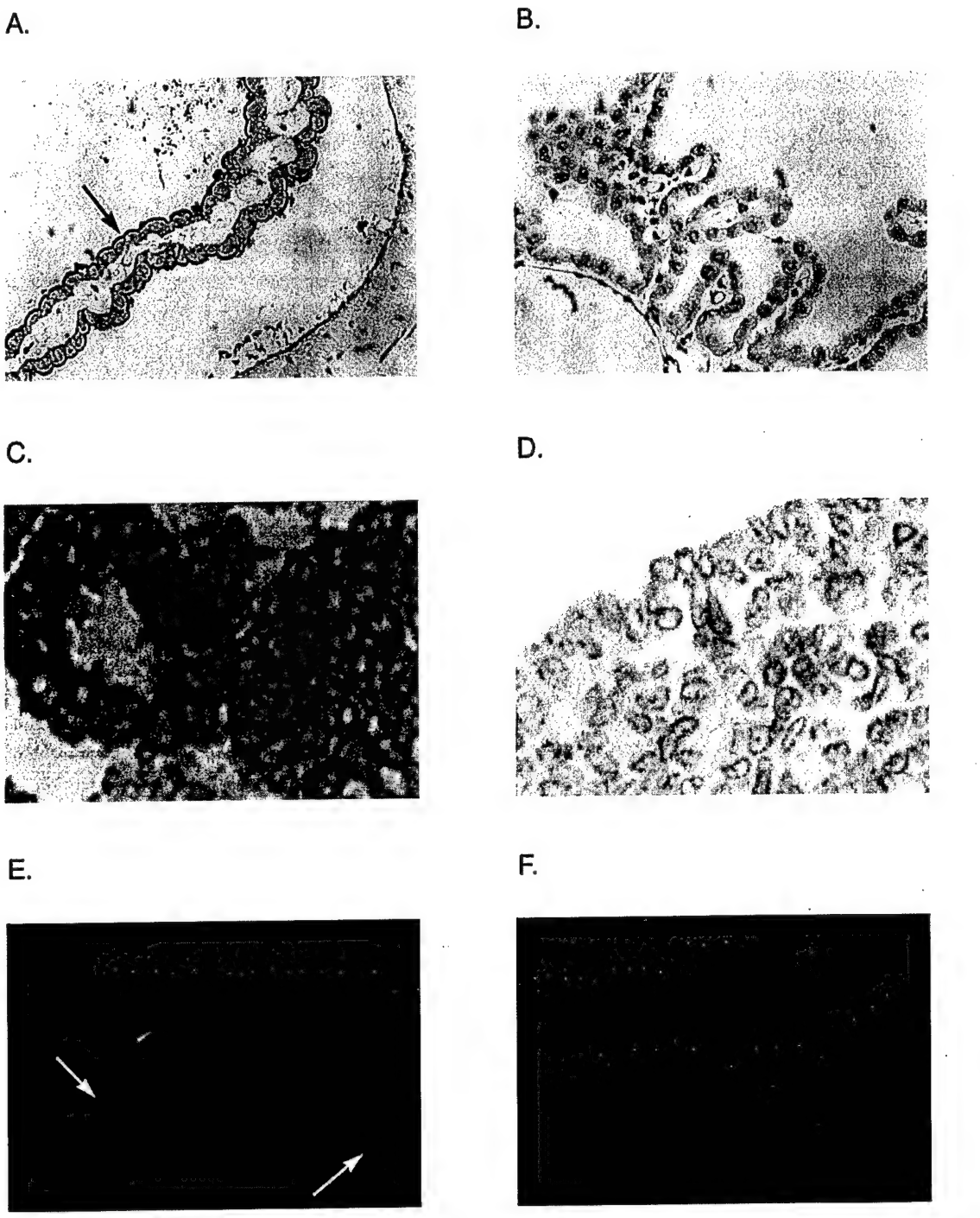
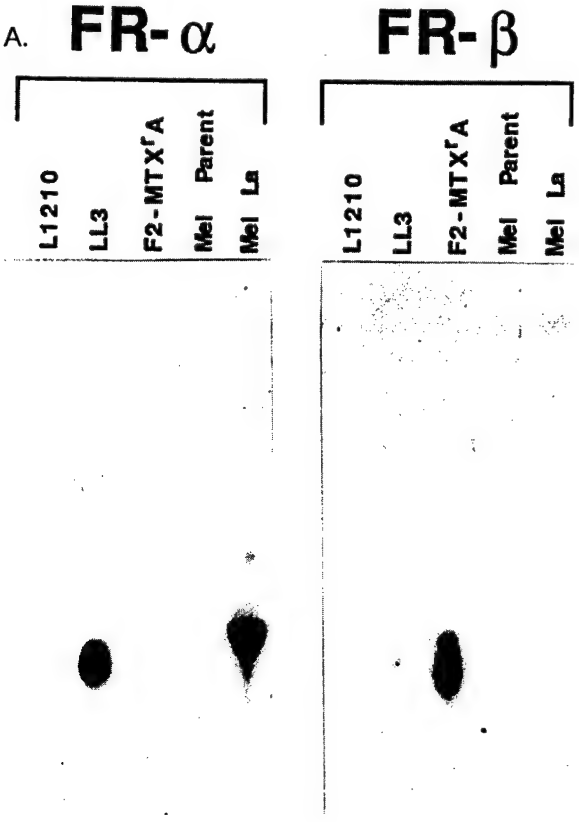


Figure 3. Immunohistochemistry of folate receptor in normal choroid plexus and choroid plexus tumor (A–D). A polyclonal rabbit antibody against the mouse folate receptor was used on AZF-fixed, paraffin-embedded tissue sections with the ABC method and metal-enhanced DAB as chromagen. A. Normal choroid plexus (1 : 5,000 dilution of primary antibody). Arrow indicates strong reactivity of apical surface of choroid plexus. B. Normal choroid plexus processed as in A. but with pimary antibody omitted as a control for nonspecific binding. C. Choroid plexus tumor (1 : 5,000 dilution of primary antibody). D. Tumor section with primary antibody omitted. E. Evans Blue dye analysis of accessibility of tissue by blood-borne agent. Staining is primarily in stromal side of normal choroid plexus. Arrows indicate apical surface of choroid plexus and normal brain parenchyma, which are unstained. F. Choroid plexus tumor of same mouse as in E., showing intense staining throughout tumor.

and hybridized with FR-specific probes as described above. Probe for β-actin was isolated from the product of an RT-PCR reaction on murine erythroleukemia cells (Mel). Membranes were washed as described above. Membranes were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) and visualized on a PhosphorImager (Molecular Dynamics).

Blood-Cerebrospinal Fluid (CSF) barrier assessment

Evans Blue is a vital dye commonly used to assess the intactness of the blood brain barrier [32]. To compare the access of blood-borne substances to tumor versus components of normal choroid plexus, 100 µl of 5% (w/v) Evans Blue (Sigma Chemical, St. Louis, MO) in 10 mM sodium phosphate 150 mM NaCl (PBS) was injected intraperitoneally. Twenty-four hrs later mice were perfused with heparinized saline followed by AZF fixative for 10 min. Brains were stored in AZF for 2 hrs, then placed in cold 15% sucrose overnight. Brains were sectioned on a cryostat at 6–20 µm, coverslipped and immediately examined by fluorescence microscopy using a rhodamine filter.



Results

To determine whether choroid plexus tumors express a protein with the properties of folate receptors, tumor membranes were examined by Western blotting with an antiserum that was raised against purified mouse FRs [33]. Flow cytometric analysis was used to confirm the specificity of this antiserum, which binds to FR-positive cell lines (LL3, F2-MTXrA, and Mel La) but not FR-negative cell lines (L1210 and Mel) (data not shown).

A major immunoreactive protein of 38 kDa was identified in the tumor but not in brain cortex that surrounded the tumor (Figure 1). This component corresponds to the 38 kDa FR previously identified on mouse cell lines by affinity labelling with NHS-[^{3}H]-folic acid [33]. A less prominent band of ap-

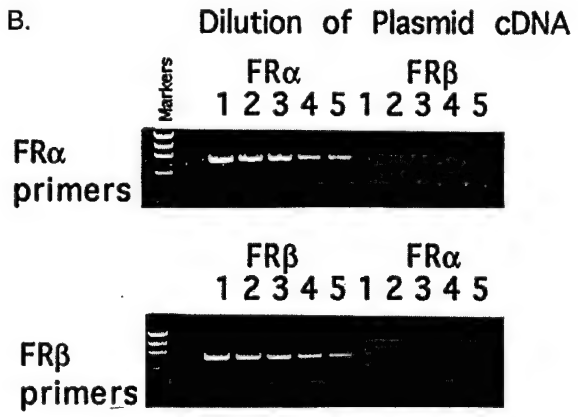


Figure 4. Specificity of probes and primers used in the detection of FR-α and FR-β transcripts. *A*. Probes. [α-^{32}P]-labeled probes to FR-α and FR-β were tested in Northern blots of mRNA from previously characterized murine cell lines. *B*. Primers. PCR primers for FR-α and FR-β were reacted with plasmid containing cDNAs for FR-α and FR-β, serially diluted by ten-fold (5 ng – 500 fg for lanes 1–5), demonstrating specificity over a five order of magnitude range.

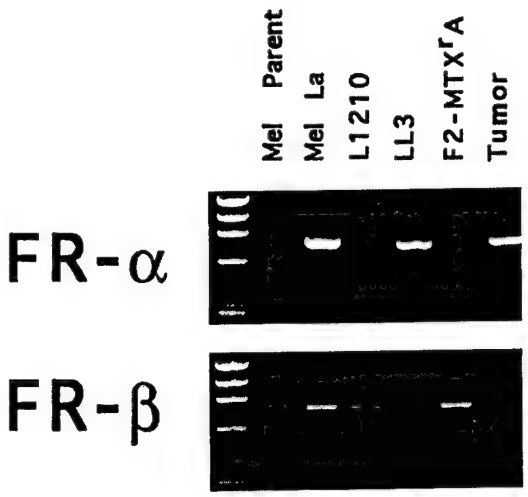


Figure 5. Detection of FR-α and FR-β transcripts in mouse tumor cell lines and tumor by RT-PCR. Total RNA from cell lines and tumor (5 μg from each) was reverse transcribed to generate cDNA. An aliquot from each sample cDNA reaction (1 μl) was amplified for FR-α or FR-β by PCR (35 cycles) using primers specific to each respective isoform. FR-α transcript is detectable by RT-PCR in L1210, LL3, Mel La and tumor as a 765 bp band. FR-β transcript is present in L1210, LL3, F2-MTX^{r}A, Mel La, and tumor as a 753 bp band.

proximately 30 kDA was also detected in tumor samples. This component corresponds to the nonglycosylated form of the FR, as previously identified by Brigle et al. [33].

Autoradiographic analysis of [^{125}I]folic acid binding to tissue sections of tumor and normal brain parenchyma showed that binding of labeled folic acid was high in the tumor (Figure 2A). Quantitation of silver grain density over tumor tissue and nonlinear regression analysis of the saturation curve indicated that the folate binding by the tumor is of high affinity, with a Kd of 1 nM (Figure 2B).

Regions of normal choroid plexus epithelium were also observed to bind significant amounts of labeled folic acid in both tumor bearing SV11 animals and C57 controls. Normal brain parenchyma had very little binding of labeled folic acid, with less than 1% of the silver grain density of the tumor or normal choroid plexus.

Immunohistochemical staining of normal murine choroid plexus with anti-FR antibody showed a highly polarized distribution of FR (Figure 3A). Reactivity was much more intense on the apical surface of the choroid plexus epithelium. In addition, a dilution of primary anti-FR antibody also demonstrated a marked disparity in the density of FR on the apical surface compared to the basal surface. For example, staining of the basal surface was barely noticeable at a 1 : 5,000 dilution of primary antibody (Figure 3A), yet staining of the apical surface persisted even at a 1 : 50,000 dilution of antibody (data not shown). The normal ependymal lining of the ventricles was also lightly stained, but FR was not detectable in normal brain parenchyma.

Immunohistochemical analysis of tumor tissue indicated a diffuse staining of FRs over the entire cell surface (Figure 3C). Since tumors in the SV11 mice arise from choroid epithelium, the FR staining pattern suggests that the individual neoplastic cells lose their original polarity. Qualitative analysis at various antibody concentrations indicated that FR density is greater on the apical surface of normal choroid epithelium than on the tumor cells (for example, at a 1 : 5,000 dilution, nearly all tumor cells were stained, but at 1 : 50,000 only rare tumor cells were stained even though the apical surface of choroid plexus was stained).

Determination of FR probe and primer specificity

The approaches described above do not distinguish between the α and β isoforms of the FR. To examine whether either α or β or both forms are expressed by tumor, RT-PCR of tumor and normal choroid plexus was performed. The specificities of the primers for PCR and the probes used in the post-PCR Southern blots were evaluated. Northern blot analysis was performed with FR cDNA probes to establish probe specificities. Transcripts for FR-α were detected in the LL3 and Mel La cell lines (Figure 4A). LL3 and Mel La are cell lines known to express the FR-α isoform [13]. Tanscripts from the two cells lines were of expected size (approximately 1700 bp for LL3 and ~ 2000 bp for Mel La). The transcript for FR-β was detected in the cell line F2-MTX^{r}A at an expected size of approximately 1600 bp (Figure 4A).

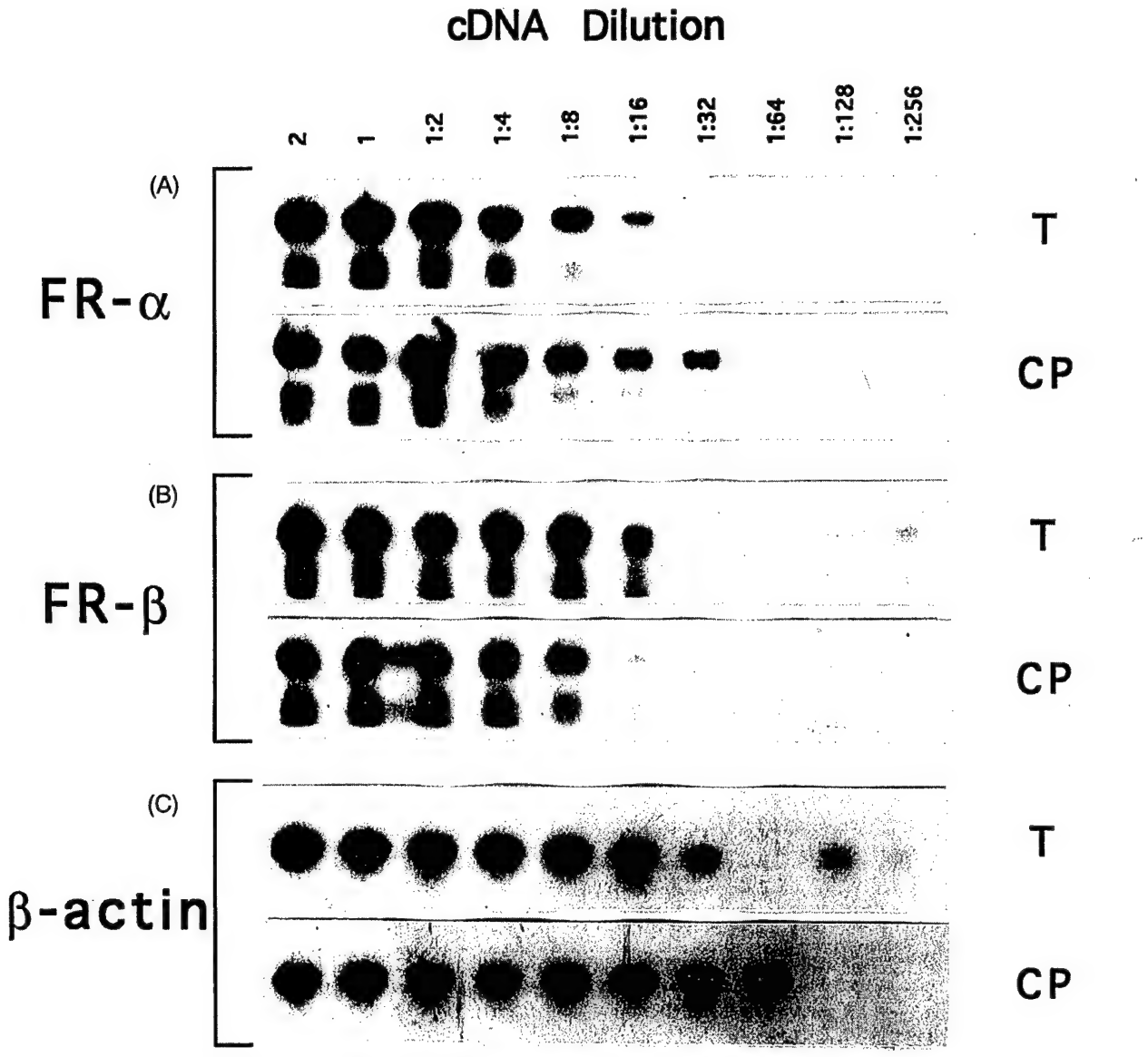


Figure 6. Detection of FR-α and FR-β transcripts in mouse choroid plexus tumor and normal choroid plexus by RT-PCR and Southern blot. Total RNA was isolated from choroid plexus (tissue pooled from eight mice) and tumor (0.5 μg RNA from each) by differential centrifugation, and reverse transcribed to generate cDNA. Aliquots of the cDNA reactions (2 μl and dilutions to 1/256 μl) were amplified for FR-α, FR-β, or β-actin by PCR (35 cycles). Amplified products (10 μl) were electrophoresed on a 1.5% agarose gel, transfered to a nylon membrane, and detected with an [α-^{32}P]-labeled probe to FR-α (*A*), FR-β (*B*), or β-actin (*C*). Membranes were exposed for 44 hrs. Counts were quantitated on a PhosphorImager following a 12 hr exposure.

For PCR, primer pairs for the coding region of each FR isoform were synthesized and their sensitivity and specificity examined. Various amounts of plasmid containing cDNA for each FR isoform (5 ng – 500 fg) were amplified by PCR in the presence of each set of FR primers. An aliquot of each

reaction product was electrophoresed in an agarose gel and visualized by ethidium bromide staining. Each primer pair was found to be sensitive to at least 500 fg and specific to amplifying its respective isoform at 5 ng (Figure 4B). Additional experiments have shown these primers to be specific up to 1 μg of cDNA-containing plasmid (data not shown). Thus the specificity of the primers extends over at least six orders of magnitude.

Detection of FR-α and FR-β transcripts in tumor and choroid plexus by RT-PCR

Due to the very small quantities of total RNA derived from choroid plexus (2 μg) and the relative insensitivity of Northern blots, RT-PCR was utilized to examine FR isoform expression in choroid plexus and tumor. The RT-PCR assay was tested on cDNA derived from five cell lines expressing varying amounts of the FR isoforms. Consistent with the Northern blot analysis, FR-α was present in LL3 and Mel La (Figure 5). In addition, L1210 also contained a detectable amount of FR-α transcript (Figure 5). FR-β was detected in all cell lines examined except the parental Mel line which is known not to express FRs of either isoform (Figure 5). Both FR-α and FR-β were detectable in tumor (Figure 5). RT-PCR followed by Southern blotting of the PCR reaction products detected both α and β FR isoform transcripts in tumor and in normal choroid plexus (Figure 6).

Analysis of the blood-CSF barrier

Because the immunoreactive FR staining of the apical surface of normal choroid plexus brought into question the usefulness of the FR as a target on tumor cells, we investigated the status of the blood-CSF barrier in SV11 mice. In non-tumor-bearing mice, macroscopic and microscopic observation of Evans Blue dye indicated that the blood-brain and blood-CSF barriers were intact (data not shown). The median eminence of the hypothalamus, a region of the brain where the BBB is incomplete, showed intense staining.

In tumor-bearing SV11 mice, fluorescence microscopic analysis of the blood-CSF barrier revealed that the stroma of normal choroid plexus stained intensely with Evans Blue, reflecting the rich supply of capillaries. The choroid epithelium itself displayed a moderate degree of staining, but the apical surface was not stained (Figure 3E). CSF and adjacent brain regions lacked staining, indicating that the blood-CSF barrier is still intact in nonaffected regions of the choroid plexus. Microscopic analysis showed intense staining throughout the tumors (Figure 3F).

Discussion

In this report, endogenously arising brain tumors of SV40 transgenic mice were evaluated for the expression of a recently described tumor-associated antigen, the high affinity folate receptor. Various independent approaches were used to show that these tumors express high affinity folate receptors with properties that are similar to the human receptor. These properties included its molecular weight ($\sim$ 38 kDa) and a binding affinity (K_D) for folate of $\sim$ 1 nM. RNA transcripts of both FR-α and FR-β isoforms were detected in the tumor.

The interest in FR expression by the SV-40-induced tumors stems from several recent findings regarding the use of FR as a target for therapy. First, human choroid plexus tumors and ependymomas, which may have a similar etiology to the transgenic mouse tumors, express FR at substantial levels [11, 12]. Thus, it seemed possible that mouse choroid plexus tumors may also express FR. Second, monoclonal antibodies to the human FR have recently been studied as possible therapeutic agents either as immunotoxins, radiolabelled antibodies, or bispecific antibodies that target tumor cells for lysis by cytotoxic T lymphocytes [28, 29]. Some of these agents have already reached clinical trials with ovarian carcinomas, of which approximately 98% express FR. We have also recently developed a novel targeting agent that consists of folic acid directly conjugated to an anti-T cell receptor antibody [30]. Finally, the endogenously derived-SV40 tumors can be used as a model that more closely mimics the human disease

than do animal studies that involve human tumor transplantation into immune deficient mice.

As with other tumor-associated antigens, the usefulness of FR as a therapeutic target depends in part on its expression in normal tissue. Earlier work with human tissues has indicated that FR-α is expressed to some degree in placenta, epithelia of the choroid plexus, epididymus, lung, thyroid, and kidney proximal tubules, ductal epithelia of the breast and pancreas, and acinar cells of the breast and salivary gland [11, 12, 24]. In the present study, *in situ* analysis revealed high levels of folate binding activity and FR not only in tumor cells but in normal choroid plexus of both control mice and SV11 mice.

One of the findings described here lends support to the notion that FR on tumor cells may be a useful target despite the presence of FR in normal choroid plexus. Immunohistochemistry shows that the FR expressed by choroid plexus is highly polarized. The protein is present on the apical surface of choroid plexus cells on the CSF side of the blood-CSF barrier. This surface may be less accessible than the tumor to blood-borne therapeutic agents. This possibility is supported by evidence that Evans Blue dye was able to stain cells throughout tumor and the stroma of the choroid plexus, but not the apical aspect of the choroid plexus epithelium.

Another possible approach to targeting tumor cells, rather than normal choroid plexus, would involve identifying whether the FR isoforms are differentially expressed. In this regard, the present results are the first characterization of isoforms of FR in any normal murine tissue. FR-α and FR-β were detected in both tumor and normal choroid plexus, but there appeared to be at least a several-fold higher expression of FR-β mRNA in tumor than FR-β mRNA in choroid plexus, based on densitometry of the probe signal and standardization to the β-actin signal. The levels of cell surface FR-α and FR-β protein on tumor cells and choroid plexus remain to be determined when appropriate monoclonal antibodies that distinguish the murine isoforms become available. However, the suggestion that FR-β may be relatively higher in some tumor cells may warrant the search for agents that target this form specifically.

Recently there have been a number of possible therapeutic agents developed that could target cells that bear folic acid receptors without the use of monoclonal anti-FR antibodies. These include folic acid analogs and folic acid conjugates that are directly cytotoxic or that deliver cytotoxic agents [26, 27, 30]. Preliminary results with a folic acid/antibody conjugate injected intravenously into SV11 mice showed that it accumulated preferentially in the tumor as opposed to other regions of the brain (E.J.R., T.A.P., D.M.K., unpublished data). This agent, along with other folic acid conjugates [34], can be used in the SV11 mice to test the relative effectiveness and potential toxicity of various therapeutic regimens.

Acknowledgements

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Single-Chain Fv/Folate Conjugates Mediate Efficient Lysis of Folate-Receptor-Positive Tumor Cells

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Single-Chain Fv/Folate Conjugates Mediate Efficient Lysis of Folate-Receptor-Positive Tumor Cells

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Bispecific antibodies that bind to a tumor antigen and the T cell receptor (TCR) redirect cytotoxic T lymphocytes (CTL) to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the folate receptor (FR), is expressed on most ovarian carcinomas and some types of brain cancer. Recently, it was shown that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high-affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein. In this paper, it is shown that the size of these conjugates can be reduced to the smallest bispecific agent yet described (30 kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody KJ16. The scFv/folate conjugates are as effective as IgG/folate conjugates in mediating lysis of FR^+ tumor cells by CTL. The optimal folate density was in the range of 5−15 folate molecules per scFv or IgG molecule, which yielded half-maximal lysis values (EC_{50}) of approximately 40 pM (1.2 ng/mL for scFv). Finally, the scFv/folate conjugates could efficiently target tumor cells even in the presence of free folic acid at concentrations that are normally found in serum. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors and in reduced immunogenicity.

INTRODUCTION

It has been known for over 50 years that the immune system is capable of attacking and eliminating very large tumor burdens but sometimes fails to do so (*1*). Although the basis of this "escape" is incompletely understood (*2*), one mechanism involves the failure of tumor cells to express antigens in a context that is essential for recognition by the immune system [reviewed by Pardoll (*3*)]. Another mechanism might be the loss of costimulatory ligands and adhesion molecules that aid in the recognition and activation of T cells (*4*).

One potential way to direct T cells or other immune effector cells against tumor cells is with bispecific antibodies [reviewed by Fanger (*5*)]. Bispecific antibodies can be constructed to recognize two separate antigens, one on the tumor surface and the other on the surface of a cytotoxic T cell (e.g. TCR[1]). Many tumor cells have potential target antigens that are tumor-specific or quantitatively more abundant on tumor cells than normal cells (tumor-associated). By bringing together the tumor cell and an activated T cell, bispecific antibodies can redirect the cytotoxicity of T cells against tumors. Previous work has demonstrated the effectiveness of bispecific antibodies against tumors *in vitro* and *in vivo* and some clinical trials have been initiated (e.g. see refs 6−12). It has generally been agreed that optimizing the properties of bispecific antibodies should improve their clinical effectiveness.

Among the tumor antigens targeted with bispecific antibodies has been the high-affinity folate receptor (FR), also called the folate binding protein. The FR is now known to be expressed at elevated levels on many human tumors, including ovarian carcinomas [e.g. one study showed that 98% of ovarian tumors express the FR (*13*)], choroid plexus carcinomas, and ependymomas (*14*, *15*). These cancers affect a significant segment of the population: ovarian cancer is the fourth leading cause of cancer death among women (*16*) and at least 30% of early childhood tumors are diagnosed as ependymomal or choroid plexus tumors (*17*, *18*).

The presence of high levels of FR on human tumor cells has made it an attractive candidate for tumor-specific therapeutics. Monoclonal antibodies to the human FR have been generated and shown to be effective at targeting FR^+ tumors *in vitro* (*19*−*21*). Clinical trials with radiolabeled antibodies and anti-FR/anti-CD3 bispecific antibodies have recently been initiated (*9*, *10*, *12*). Another approach has been to use the endocytic properties of the FR to deliver toxins or antisense nucleotides to the interior of malignant cells (*22*, *23*). Although relatively low levels of FR mRNA have been detected in most normal human tissues (*14*), several studies have shown that normal choroid plexus, kidney, thyroid, colon, and placenta may have elevated levels [*14*, *24*, *25*; reviewed by Antony (*26*)]. Despite the presence of FR on normal human tissue, clinical trials using the anti-FR/anti-CD3 bispecific antibody have not demonstrated any toxicity associated with cytolysis of normal FR expressing tissue (*9*, *10*).

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[1] Abbreviations: CTL, cytotoxic T lymphocyte; FR, folate receptor; TCR, T cell receptor; scFv, single-chain antibody binding domain; MHC, major histocompatibility complex; K_D, dissociation constant; SV40, simian virus 40; EC_{50}, concentration of antibody/folate conjugate required for half-maximal CTL mediated cytotoxicity; fol, folate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; FITC, 5-aminofluorescein isothiocyanate; Ga, gallium; Fab, antigen binding fragment derived from papain digestion of Ig molecule; V regions, variable regions of IgG heavy and light chains; ϵ, extinction coefficient; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.3; PBS-BSA, PBS containing 0.1% bovine serum albumin; MTX, methotrexate.

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The high intrinsic affinity of folate for FR ($K_d \sim 1$ nM) suggested to us that attachment of folate directly to an anti-TCR antibody might efficiently target FR^+ tumor cells for lysis by T cells. We recently reported that such conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein [$K_d \sim 1.5 \times 10^{-6}$ (*27*)], which is responsible for normal dietary uptake of folate and the transport of folate-based dihydrofolate reductase inhibitors such as methotrexate. It is reasonable to predict that the most effective agents for targeting solid tumors will have reduced sizes that allow greater tumor penetration. For instance, comparative biodistribution studies with ^{125}I-labeled IgG, $F(ab)_2$, Fab fragments, and scFv in human colon carcinoma xenografts in athymic mice demonstrated that scFv molecules penetrated tumor more rapidly, to a greater depth, and more uniformly than other forms of the antibody (*28*).

In this paper, a 29-kDa scFv of the anti-Vβ8 antibody KJ16 (*29*) was conjugated with folate and its targeting potential was evaluated *in vitro*. Cytotoxicity assays with these preparations showed that lysis of mouse FR^+ tumor cells was highly specific and correlated directly with FR density ($r = 0.93$). Comparison between folate-labeled-IgG and scFv demonstrated that both conjugates have nearly identical targeting efficiencies ($EC_{50} = 40$ pM), and lysis with scFv/folate could be detected at concentrations as low as 1 pM. Direct competition experiments with free folate demonstrated that the scFv/folate conjugate could effectively target FR^+ tumor cells even at folate concentrations above normal serum levels. The reduced size of the scFv/folate compared to other bispecific reagents as well as its high potency suggests that it has potential for *in vivo* therapy. In addition, the conjugate may serve as a model for the development of future novel bispecific agents that contain small ligands specific for tumor cell surface antigens.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies. The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal calf serum, 1.3 mM L-glutamine, 100 units of penicillin/mL, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol: Mel, murine erythroleukemia cell (*30*); La, a subline of Mel selected on low folate (*31*); L1210, a murine leukemia cell line (*32*); and F2-MTX^{r}A, a MTX-resistant subline of L1210 selected for increased expression of FR-β by growth on low folic acid (*33*). La expresses primarily the α isoform of folate receptor (FR), F2-MTX^{r}A expresses only the FR-β isoform, and L1210 expresses both α and β isoforms. CTL clone 2C, a $V\beta8^+$ alloreactive cell line specific for L^d (*34*), was maintained in the same RPMI medium supplemented with 10% (v/v) supernatant from concanavalin A-stimulated rat spleen cells, 5% methyl α-mannoside, and mitomycin C treated BALB/c mouse spleen cells as stimulators. KJ16 is a rat IgG antibody specific for the Vβ8.1−2 domains of the TCR (*35*) and was provided by Drs. Kappler and Marrack. KJ16 monoclonal antibody was prepared from tissue culture supernatant generated in a Vita-Fiber miniflow path bioreactor (Amicon) and concentrated by precipitating twice in 50% ammonium sulfate. KJ16 Fab fragments, FITC-labeled Fab fragments, and KJ16 scFv were generated and purified as described previously (*29*). Briefly, scFv was refolded from inclusion bodies, and monomeric scFv was purified by G-200 HPLC purification. Monoclonal antibody 30.5.7 is specific for the major histocompatibility complex (MHC) class I product L^d (*36*) and was prepared as ascites fluid and used without further purification in some cytotoxicity assays.

Preparation of Antibody/Folate Conjugates. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously (*27*, *37*). Unless indicated, a 5-fold molar excess of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, Pierce Chemical Co.) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 min at room temperature in the dark, a 20−700-fold molar excess of the EDC-activated folate was added to 0.1−0.5 mg of antibody in 0.1 M MOPS, pH 7.5. After 1 h at room temperature, the sample was either applied to a Sephadex G-25 column pre-equilibrated in 0.1 M MOPS or immediately dialyzed into phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3). If passed over a G-25 column, the excluded-peak fractions were pooled and dialyzed against PBS. After dialysis, protein concentrations were determined using the bicinchoninic assay (Pierce) using a protein A purified intact mouse antibody as the standard. Antibody conjugates were also analyzed spectrophotometrically at 363 nm, and the density of the folate per antibody was calculated by dividing the molar concentration of folate on the conjugate ($A363/\epsilon_M$; $\epsilon_M = 6197\ M^{-1}$) by the antibody concentration. Folate densities obtained ranged from ~1 to ~120 folates/antibody. Conjugates were stored at 4 °C in the dark.

Mass Spectrometry. Mass spectra were obtained on a TofSpec using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and concentrated to 10−25 pmol/mL. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois.

Folate Binding Assays. Binding assays were conducted by using ^{125}I-labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.3 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 50 μL of PBS-BSA for 1 h at 37 °C. Incubation at 37 °C has previously been shown to produce levels of binding similar to that obtained with acid pretreatment (*27*). Samples were loaded into tubes containing 300 μL of oil [80% (v/v) dibutyl phthalate/20% (v/v) olive oil], and bound and free ligand were separated by a 3 s centrifugation at 12000*g*. Tubes were frozen and cut to allow the radioactivity in the cell pellet and supernatants to be quantitated separately.

T Cell Receptor Binding Assays. The relative affinity of the scFv/folate conjugates for the TCR was determined by a competition assay with 5-aminofluorescein isothiocyanate (FITC)-labeled KJ16 Fab fragments as previously described (*29*). In brief, various concentrations of antibody were added to triplicate sets of 6×10^5 2C cells in the presence of a constant amount of FITC-labeled Fab fragments. After a 30 min incubation on ice, the entire mixture (antibody + FITC-labeled Fab fragments + 2C cells) was passed through a flow cytometer without washing. Inhibition by various KJ16 preparations was measured by quantitating the decrease in bound fluorescence by flow cytometry (performed with a Coulter Electronics EPICS 752 at the University of Illinois Biotechnology Center). The concentrations of unlabeled antibody giving 50% inhibition (IC_{50}) were determined relative to the maximum fluorescence (in the absence of inhibitor) and the background fluorescence (in the presence of a large excess of intact antibody).

Cytotoxicity Assays. Tumor cells were labeled with

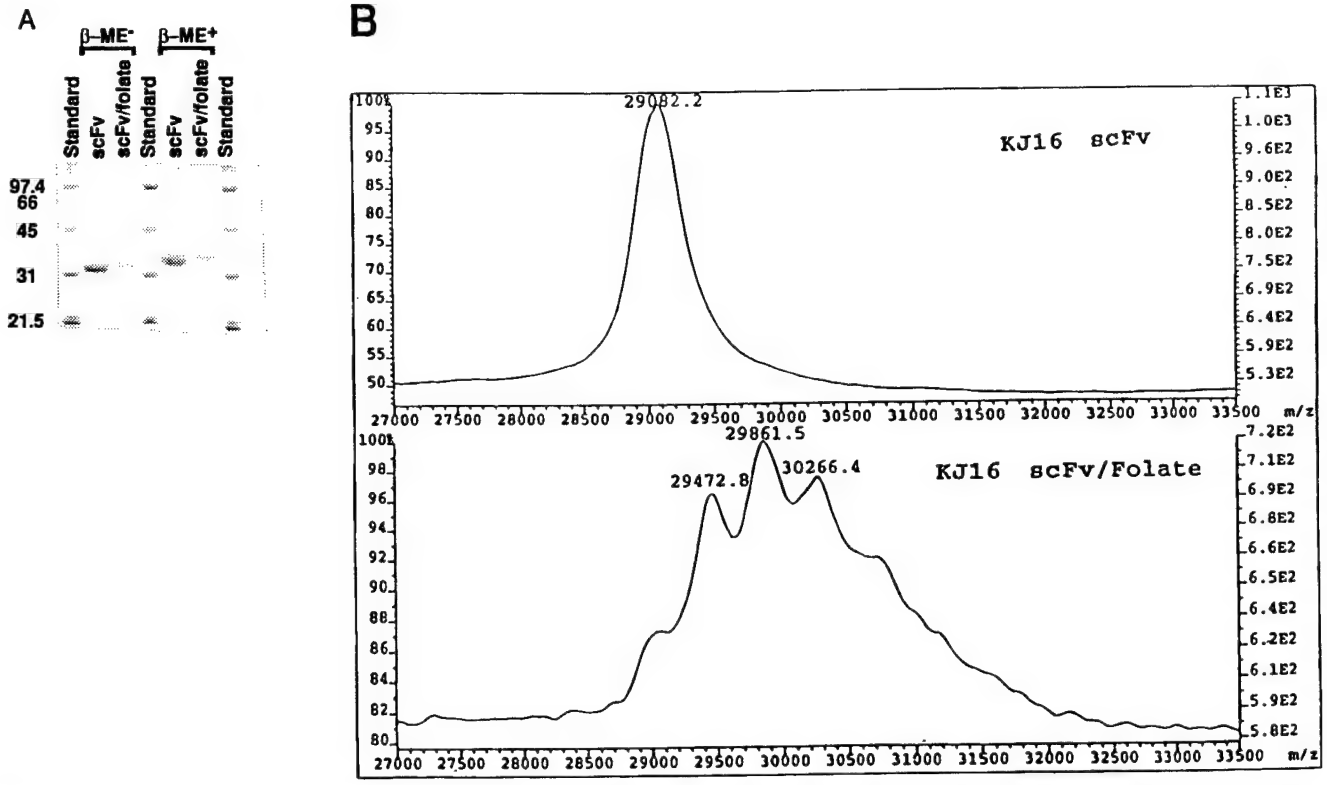

Figure 1. SDS–PAGE analysis and mass spectra of purified preparations of scFv KJ16 and scFv/folate conjugate: (A) samples were electrophoresed through a 10% polyacrylamide gel under reducing and nonreducing conditions, and proteins were visualized by staining with Coomassie Blue; (B) samples were concentrated, dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and mass spectra were obtained on a TofSpec using electrospray ionization. Purified scFv existed as a single species with a molecular mass of 29 082 Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular mass of a folate molecule (~400 Da), detectable up to 7 folates per antibody.

50–100 μL of ^{51}Cr (2.5 mCi/mL) for 60 min at 37 °C, washed twice with folate-free RPMI 1640 medium containing 5% (v/v) fetal calf serum (folate-free media), and used in 96-well plate cytotoxicity assays at 10^4 cells per well. Because each of these cell lines also expressed the alloantigen L^d, which is recognized by CTL 2C, assays were performed in the presence of anti-L^d antibody to minimize non-FR-mediated lysis. Ascites of anti-L^d antibody 30.5.7 was diluted 1:100 into folate-free media containing effector cells (2C). Effector cells were added to target cells at an effector-to-target cell ratio of 5:1. Antibodies and folate/antibody conjugates were diluted in folate-free media and added to triplicate wells at various concentrations. Plates were incubated at 37 °C for 4 h in 5% CO_2, and supernatants were removed for γ counting. For the inhibition of scFv/folate by free folate, a nonsaturating concentration of scFv/folate (3 nM) that would generate maximal cytotoxicity was used together with various concentrations of free folate. Unless indicated otherwise, the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates [e.g. % specific release = (experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100].

EC_{50} values (i.e. the concentration of antibody/folate conjugate required for half-maximal specific lysis) were determined by linear regression. Among independent experiments, EC_{50} values and maximum cytotoxicity could vary for the same conjugate preparation primarily due to differences in CTL activity. For example, noticeable reductions in CTL 2C activity can be observed after multiple passages *in vitro* (unpublished data, D.M.K.). Therefore, for comparison of EC_{50} values from different experiments (i.e. Figure 5B), assay results were normalized by dividing each calculated EC_{50} by the EC_{50} of the most potent conjugate in a given experiment [e.g. $EC_{50}^{normalized} = (EC_{50}/EC_{50}^{low})$]. The inverse of this normalized value, $[(EC_{50}^{normalized})^{-1}$, which we have called the targeting index] was plotted as a function of folate density on the antibody, where a targeting index = 1 specifies the most potent conjugate.

RESULTS

Characterization of scFv/Folate Conjugates. The scFv of KJ16, an anti-Vβ8 antibody, was purified from *Escherichia coli* inclusion bodies after guanidine denaturation, refolding, and HPLC gel filtration. Purified scFv migrated as an apparent 35-kDa protein on SDS–PAGE gels (Figure 1A). Folate was coupled to the scFv using the carbodiimide (EDC) reaction, which links carboxyl groups of folate to primary amine groups on the protein. In the engineering of the scFv, the V_L and V_H domains were joined by the 26 residue linker, 205s, that contains 8 lysine residues (*29*, *38*). We reasoned that the presence of multiple lysine residues in a highly accessible, solvent-exposed region may result in higher folate densities in the linker region as opposed to the antibody V regions. In initial studies, folate was coupled to the scFv at a 100:1 folate to antibody molar ratio. Under these conditions, scFv/folate preparations contained an average of 3–8 folates per antibody (N = 3), on the basis of spectrophotometric analysis. Consistent with this finding, migration of the scFv/folate conjugate on SDS–PAGE gels was slightly slower than that of scFv and the band was more diffuse (Figure 1A).

The folate density determined by photometry does not provide information about the heterogeneity of the conjugates. To determine the range of epitope densities within a single preparation, scFv/folate conjugates were

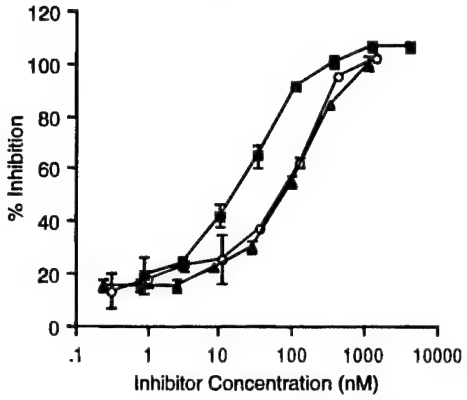

Figure 2. Binding of KJ16 scFv/folate to cell surface T cell receptor. The binding of FITC-labeled KJ16 Fab fragments to the Vβ8-positive T cell clone 2C was inhibited by purified scFv/folate, scFv, or Fab fragments. A total of 6×10^5 2C cells was incubated for 30 min at 4 °C with FITC-labeled KJ16 Fab fragments ($\sim 7.0 \times 10^{-8}$ M) and various concentrations of folate-labeled scFv KJ16 (7.8 fol/scFv, ▲), unlabeled scFv (■), and unlabeled Fab fragments (○). A relative affinity of the scFv/folate was determined by comparing the concentrations required to inhibit 50% of the FITC-labeled Fab fragments from binding the 2C TCR.

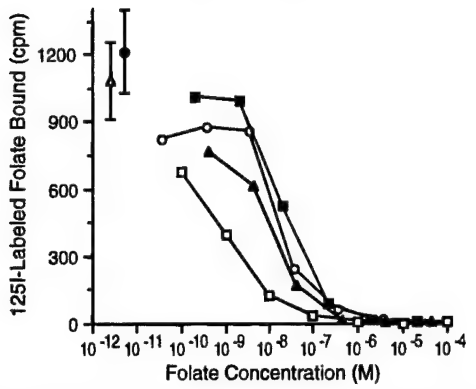

Figure 3. Binding of KJ16 scFv/folate to folate receptors. ^{125}I-labeled folate ($\sim 1.8 \times 10^{-10}$ M, 2000 Ci/mM) was incubated with F2-MTX^{r}A cells in the presence or absence of competitors for 1 h at 37 °C. Concentrations refer to folate rather than antibody concentrations. Competitors included free folate (□) and KJ16 scFv/folate conjugates with different folate densities: 2.8 fol/scFv (○), 9.2 fol/scFv (■), and 20.4 fol/scFv (▲). Inhibition was not observed in the absence of competitor (△) or in the presence of unconjugated scFv (●) (error shown is ± average SEM).

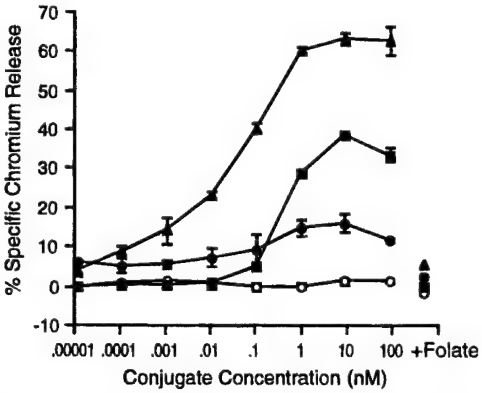

Figure 4. Cytotoxicity assay of various tumor cells lines with the scFv/folate conjugate and CTL clone 2C. Various concentrations of the scFv/folate conjugate were incubated with ^{51}Cr-labeled tumor cells and CTL 2C for 4 h at an effector-to target ratio of 5:1. Experiments were performed in the presence of anti-L^d antibody to minimize lysis due to recognition of L^d, the nominal ligand for CTL 2C. Lysis correlated directly with the level of FR expressed by the cell line: F2-MTX^{r}A (200 000 sites/cell, ▲); La (60 000 sites/cell, ■); L1210 (8 000 sites/cell, ●); Mel (not detectable FR, ○). Assays with free folate at a final concentration of 1.5 μM were performed with the scFv/folate conjugate at a concentration of 0.09 nM (+ folate).

examined by electrospray ionization mass spectrometry (Figure 1B). Unlabeled scFv demonstrated a single peak with a molecular mass of 29 082 Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular mass of a folate molecule (~400 Da), detectable up to 7 folates per antibody. Integration of mass spectra showed that >85% of the scFv molecules were labeled with one or more folate molecules. Folate densities estimated from mass spectra were generally 1.5–2-fold lower than densities estimated by spectrophotometry. This could in part be due to lower solubility of high-density conjugates under conditions required for mass spectrometry or to the dissociation of some folate molecules during ionization.

Binding of scFv/Folate Conjugates to the T Cell Receptor. To examine if folate conjugation affected the binding of scFv antibodies to the TCR, a scFv/folate conjugate was compared with unlabeled scFv. In a competitive flow cytometric assay, fluorescein-labeled KJ16 Fab fragments were inhibited from binding the Vβ8-positive CTL clone 2C by unlabeled Fab fragments, scFv, or scFv/folate (Figure 2). As shown previously, scFv antibodies have an approximate 3-fold higher apparent affinity than Fab fragments, possibly because of the presence of noncovalently associated scFv dimers (*29*). Comparison of folate-labeled and unlabeled scFv showed that the folate conjugate had an apparent affinity ~3-fold lower than scFv (i.e. approximately equal to KJ16 Fab fragments). This decreased binding, compared to unlabeled scFv, could be due either to chemical modification of active site residues with folate or to the interference of dimer formation by folate. The fact that folate conjugates bound the TCR as well as KJ16 Fab fragments, which have a K_D of ~130 nM (*29*, *39*), indicated that the conjugates have potential to mediate lysis of target cells by CTLs.

Binding of scFv/Folate Conjugates to Folate Receptors on Tumor Cells. The ability of scFv/folate conjugates to bind folate receptors (FR) on the surface of tumor cells was examined by a competition binding assay using ^{125}I-labeled folate as the labeled ligand (Figure 3). The competition assay used the F2-MTX^{r}A cell line that expresses the β isoform of the FR. Competitors included various concentrations of free folate, unlabeled scFv, and three different scFv/folate preparations. Examination of the binding curves showed that folate-conjugated antibody, but not unlabeled scFv, binds to the FR$^+$ tumor cell line. However, on the basis of molar folate concentration, the folate conjugates had a relative affinity that was approximately 10–30-fold less than that of free folate. This decrease in apparent affinity was consistent with previous observations (*27*) and could be partly attributed to the carbodiimide labeling procedure. This procedure links folate through either the α or γ carboxyl group, but only linkage through the γ carboxyl retains binding (*40*). It is also possible that receptor-mediated internalization or decreased accessibility of the FR may also explain the lower apparent affinity of the folate conjugate. Included in the latter is the possibility that neighboring folates or amino acids sterically hinder interaction with the FR.

CTL-Mediated Lysis of FR$^+$ Tumor Cells by scFv/Folate Conjugates. The specificity and efficiency of tumor targeting with scFv/folate conjugates were examined in a ^{51}Cr release assay with CTL clone 2C (Figure 4). Four different tumor cell lines that have a range of cell surface FR densities were used as target cells: F2-

MTX^{r}A (200 000 sites/cell); La (60 000 sites/cell); L1210 (8 000 sites/cell); Mel (no detectable FR). Each of the FR$^+$ cell lines was lysed in the presence of the scFv/folate conjugate, and the extent of lysis was directly correlated with the expression level of the FR ($r = 0.93$). Lysis was completely inhibited by free folate, indicating that targeting of the tumor cells was specifically mediated by the folate receptor and not some other cell surface molecule. The lysis mediated by these conjugates was highly specific (e.g. the FR$^-$ cell line Mel was not lysed by the conjugate even at concentrations over 10^5 times that required for detectable killing of the FR$^+$ cell line F2-MTX^{r}A) and extremely potent (e.g. lysis was detectable at concentrations as low as 1 pM of scFv/folate). The slight reduction in observed lysis at the highest concentrations of conjugate occurs because excess bispecific antibody yields monospecific binding without cell-to-cell cross-linking (*41*). The presence of reduced folate carrier protein (as present in Mel and in all other normal cells) does not result in cell destruction. It is important to point out that the FR density reportedly on ovarian tumors is even higher (~1 million/cell) than those on these tumor cell lines (*21*).

Effects of Folate Density on Targeting. To examine the effects of folate density and labeling on the targeting efficiency of scFv/folate conjugates, the antibody was labeled with folate under various carbodiimide-mediated coupling conditions. The carbodiimide EDC couples folate through the free carboxyl groups, but when used in the presence of protein, it may also lead to protein modification and subsequent precipitation or inactivation. To evaluate the optimal levels of EDC for folate coupling, several different concentrations of EDC at a constant folate concentration were used during coupling. EDC used at either 13 or 65 mM generated conjugates with approximately equal targeting efficiency. EDC used at 260 mM yielded conjugates with reduced efficiency and frequently led to protein precipitation (data not shown).

To directly examine the effect of folate density, activated folate was prepared at a constant EDC/folate ratio (5:1) and conjugates were produced by adding different ratios of activated folate to the scFv protein. After dialysis to remove unreacted folate and excess EDC, conjugates were evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency. Folate densities ranged from approximately 1 to 20 folates per scFv. As shown in Figure 5A, each of the conjugates was capable of mediating lysis of the FR$^+$ tumor cell line by CTL clone 2C. However, conjugates with either the lowest density (1.3 folates/scFv) or highest density (20.4 folates/scFv) were 5−10-fold less effective than conjugates with intermediate folate densities.

To better evaluate the effects of folate density, the above experiment was performed with several additional folate conjugate preparations. To directly compare the results of these assays, the EC_{50} of each conjugate was determined by linear regression and divided by the EC_{50} of the conjugate that yielded the highest targeting efficiency (i.e. the lowest EC_{50}; see Experimental Procedures). A plot of the normalized targeting values as a function of the folate density (Figure 5B) demonstrates that optimal folate density appears to be in the range of 5−15 folates/scFv. At higher folate densities, the targeting efficiency of the conjugates is generally lower and more variable than those of conjugates with an intermediate amount of folate. The reduction in targeting efficiency at higher folate densities is not due to the inability of these conjugates to bind to FR$^+$ cells (Figure 3), but is likely a consequence of chemical modification

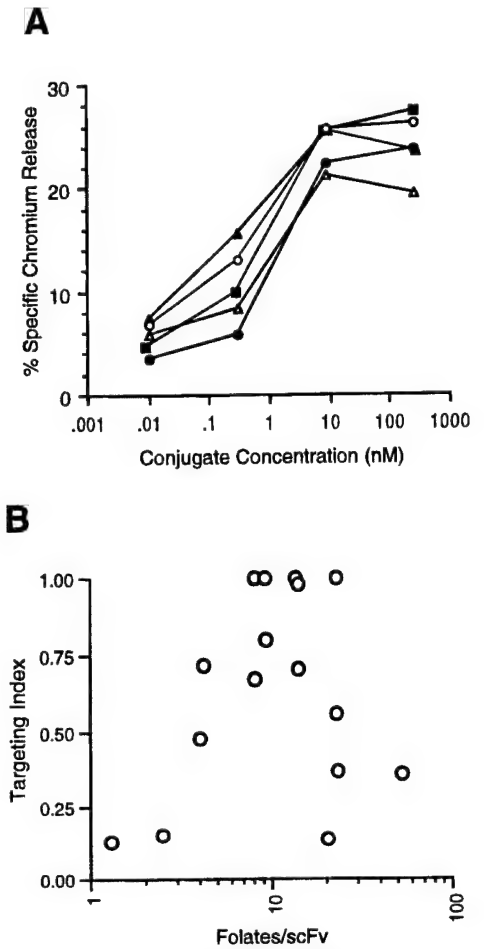

Figure 5. Effect of folate density on targeting with scFv/folate conjugates: (A) scFv/folate conjugates with different folate densities were prepared by labeling scFv protein with various amounts activated folate, using an EDC/folate ratio of 5:1 (EDC = 65 mM). Folate densities were 1.3 fol/scFv, ●; 4.0 fol/scFv, ■; 9.2 fol/scFv, ▲; 9.3 fol/scFv, ○; and 20.4 fol/scFv, △. Conjugates were tested in cytotoxicity assays using F2-MTX^{r}A and CTL 2C. (B) Relative targeting efficiency of various scFv/folate conjugates as a function of increasing folate density. Targeting index values were determined by normalizing EC_{50} values of the various conjugates with the most effective conjugate (value = 1) (see Experimental Procedures for details). Each open circle (○) represents a single scFv/folate conjugate prepared using an EDC/folate ratio of 5:1 (EDC 33−65 mM) and tested in cytotoxicity assays using F2-MTX^{r}A and CTL 2C.

of amino acid residues important in TCR binding by the scFv or in scFv stability.

Comparison of Intact Antibody and scFv. To determine the relative effectiveness of intact KJ16 IgG versus scFv-KJ16, both forms were labeled with folate at a 100:1 molar ratio of activated folate to antibody and under identical EDC reaction conditions (33 mM EDC, 6.7 mM folate). These conditions yielded folate densities of 7 and 5 for the scFv and intact antibody, respectively. Cytotoxicity assays with these preparations showed nearly identical targeting efficiencies for the intact and scFv forms of KJ16 (Figure 6). The concentration required to obtain 50% of the maximal specific release (EC_{50}) was approximately 40 pM (1.2 ng/mL for scFv). Comparison of intact and scFv conjugates in an ^{125}I-labeled folate binding assay indicated no significant difference in their ability to bind FR (data not shown).

Intact KJ16 antibody was also labeled at various folate densities to determine if targeting efficiency could be optimized further. Conjugates were again evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency (Fig-

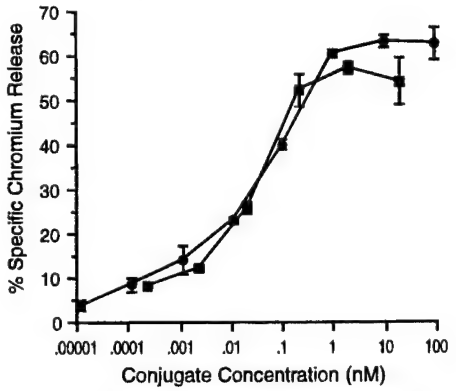

Figure 6. Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells. Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 mM EDC, 100:1 molar ratio of folate/antibody), yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C.

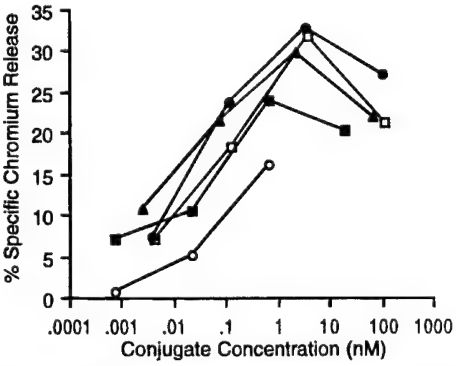

Figure 7. Effect of folate density on targeting with IgG/folate conjugates. Intact antibody was labeled at several folate concentrations to determine if, like scFv, a specific range of folate density would yield the optimal targeting effectiveness. Conjugates with the following densities were assayed with ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C: 4.5 fol/IgG, □; 7.0 fol/IgG, ●; 13.6 fol/IgG, ▲; 56.7 fol/IgG, ■; and 126.2 fol/IgG, ○.

ure 7). Folate densities ranged from approximately 4−126 folates/antibody molecule. As shown with scFv preparations, high and low folate density resulted in decreased targeting effectiveness for the intact antibody. As with scFv preparations, the optimal densities appear to be in the range of 5−15 folates/antibody, as determined by spectrophotometry.

Inhibition of scFv/Folate Conjugate-Mediated Lysis by Free Folate. Normal serum folate may reduce the effectiveness of the scFv/folate conjugate by competing for the folate receptor *in vivo*. A ^{51}Cr release assay was used to evaluate the effectiveness of the scFv/folate conjugate-mediated lysis at biologically relevant concentrations of free folate (Figure 8). Free folate was diluted in folate-free media, and various concentrations were added to triplicate wells containing ^{51}Cr-labeled F2-MTX^{r}A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (3 nM) that would generate maximal specific release. The resulting titration curve demonstrated that at normal human serum folate levels [9−36 nM (*42*, *43*)] the scFv/folate conjugate retained most of its activity. For instance, at 20 nM folate, the scFv/folate conjugate exhibited over 80% of its CTL-mediated targeting potential. Although murine serum folate levels are significantly higher than normal human serum values (124−700 nM) due to folate-rich chow (*44*, *45*), even at these elevated folate concentrations, the scFv/folate conjugate exhibited 30−60% of its targeting potential.

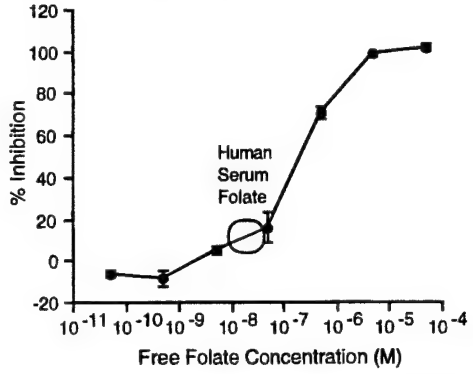

Figure 8. Inhibition of scFv/folate-mediated lysis by free folate. Various concentrations of free folate were added to triplicate wells containing ^{51}Cr-labeled F2-MTX^{r}A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (~8 fol/scFv) that would yield maximal specific release (3 nM). Inhibition was calculated as a function of the specific release in the absence of folate competitor. Normal human serum folate concentrations range from 9 to 36 nM (*42*, *43*).

DISCUSSION

The most effective agents for targeting solid tumors will likely have reduced sizes that allow greater tumor penetration. This paper characterizes the smallest bispecific agent yet described for redirecting the activity of immune effector cells against tumors. Initial bispecific antibody studies to target ovarian tumors that express high-affinity FR have used intact heterobifunctional antibodies (~150 kDa) that bind to CD3 and the FR. These agents showed efficacy in animal models, and they have recently entered testing in clinical trials (*9*, *10*, *12*). Although not yet reported for anti-FR antibodies, several laboratories have shown that it is possible to engineer smaller bispecific antibodies of ~60 kDa by linking two scFv regions (*41*, *46*, *47*). Here we show that the size of a bispecific targeting agent can be reduced even further to ~30 kDa for the scFv/folate conjugates. Furthermore, the targeting efficiency of the engineered scFv/folate conjugate is comparable to that of the native intact antibody/folate conjugates.

For coupling of folate to the anti-Vβ8 scFv KJ16, a carbodiimide reaction that links the carboxyl groups of folate to the amino groups of the antibody was used. KJ16 scFv binds to the cell surface TCR with an affinity that is similar to that of the intact KJ16 antibody (*29*). The V_L and V_H regions of the scFv are linked by a 26 amino acid region that contains 8 lysine residues. We reasoned that this charged linker would be accessible to folate, would be located distal to the binding site, and would contain multiple attachment sites for folate through the EDC reaction. Under coupling conditions where folate concentrations are nonsaturating, the scFv and intact antibody have comparable densities of folate attached per molecule (~5−15 folates/molecule), despite the 5-fold greater size of the intact antibody. Cytolytic assays demonstrated that these conjugates have very similar targeting efficiencies (Figure 6).

Although folate is probably attached to lysines present in the linker, the scFv/folate conjugates are actually heterogeneous populations as evidenced by mass spectra (Figure 1B). Within a scFv/folate preparation there are most likely conjugates with enhanced targeting properties and conjugates with diminished targeting properties. The former could include not only those with folate at an accessible location but perhaps those with multiple

folates that allow for multivalent interactions with FRs on tumor cells. Conjugates which have diminished bispecific properties would include those that have folate attached through amine groups in the scFv active site or in regions which destabalize the V_L-V_H interaction. These are analogous to those preparations derived with very high folate densities, where the targeting efficiency is significantly reduced (Figures 5 and 7).

Several considerations suggest that further optimization of the scFv/folate conjugates could yield even more potent agents. The anti-TCR antibody KJ16 has a relatively modest K_D of ~100 nM (*29*). Our laboratory is currently engineering higher affinity anti-TCR antibodies by antibody display methods; antibodies with $K_D \leq 1$ nM have been routinely generated using similar approaches (*48*). In addition, the affinity of the scFv/folate conjugate for the FR^+ tumor cells was up to 30-fold less than the affinity of free folate for the FR (Figure 3). Coupling of folate through the γ carboxyl, plus homogeneous linkage perhaps through cysteine or multiple cysteines, should improve the affinity for the FR. Alternatively, other folate analogs with higher affinity than folate could be employed (*49*). The use of these strategies should allow the development of scFv conjugates that have EC_{50} values considerably less than those described in this paper.

It is of significant note that the scFv agent described here not only is the smallest agent but is at least as potent *in vitro* as bispecific antibodies described in the literature. For example, the EC_{50} of other bispecific agents range from 1 to 100 ng/mL (*21*, *50*–*52*). Various other antibody or folate-based targeting agents have EC_{50} values that range from 0.1 to 200 ng/mL (e.g. refs 37, 40, 53–56). *In vitro* assays for these other agents typically involved 24 h incubation periods, while the cytotoxicity assays for CTL-mediated lysis, described here and elsewhere, are 4 h incubations. Thus, there is reason to believe that the scFv/folate conjugate has considerable promise *in vivo*: It has an EC_{50} of approximately 1 ng/mL, is smaller than the other agents, and remains effective at folate levels found in normal human serum. The scFv/folate targeting effect was inhibitable by free folate, but only at concentrations that were considerably higher (>1000-fold) than the folate conjugate concentration (Figure 8). Schodin *et al.* (*57*) have previously shown that <1% of the total number of TCRs per CTL need to be triggered for CTL-mediated cytolysis to occur. Thus, it is possible that >90% of the folate/antibody conjugates were inhibited from binding the target cell but sufficient conjugate remained bound to trigger maximal lysis.

The fact that the scFv/folate conjugate is in direct competition with serum folate also brings about the intriguing possibility of modulating the effectiveness of scFv/folate conjugate treatment by altering the levels of serum folate. For instance, recent studies with mice have shown that serum folate can be intentionally decreased up to 100-fold with special low-folate diets (*44*, *45*). The decreased folate concentration greatly enhanced the ability of a ^{67}Ga-labeled deferoxamine/folate conjugate to image FR^+ tumors *in vivo* (*44*). We envision that similar low-folate diets will likewise enhance the therapeutic effectiveness of the scFv/folate conjugate. Conversely, serum folate could be increased in situations where nonspecific T cell interactions lead to adverse side effects. Thus, the use of folate as the small molecule ligand specific for a tumor antigen may allow for additional levels of regulation normally not available with other immunotargeting agents.

In vivo tests to compare scFv and intact folate conjugates are currently underway in SV40 transgenic mice that develop choroid plexus tumors exhibiting elevated levels of the high affinity folate receptor (*58*). Preliminary results indicate that T cells specifically infiltrate the tumor after treatment with the scFv/folate conjugate (B.K.C., T.A.P., D.M.K., and E.J.R., unpublished data). At this time, it is unclear whether conjugate-bound T cells extravasate into the tumor or if T cells first extravasate into the tumor and subsequently recognize the conjugate bound to cell surface FR. The latter mechanism would favor the enhanced tumor penetration characteristics of the smaller scFv molecule and would likely lead to increased therapeutic effectiveness.

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Targeting Tumor Cells with Bispecific Antibodies and T Cells

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Abstract

It has been known for some time that mammalian immune systems are capable of eliminating large tumor burdens. Redirecting the immune response of a patient to an established tumor has now become the focus of various therapeutic strategies. In this report, two projects toward this goal are described. The first project involves the development of a transgenic mouse model for T cell directed therapeutics. These mice express specific T cell receptor α and β transgenes on a background in which the recombinational-activating-gene-1 (RAG) has been knocked out. The mice express cytotoxic T cells but not either T helper cells or B cells. Despite these deficiencies, the animals are capable of eliminating tumors that express the appropriate peptide/major histocompatibility complex ligand that is recognized by the $\alpha\beta$ transgenic T cell receptor. Human tumors grow as transplants in these mice, thereby allowing various agents that redirect the endogenous T cells against human tumors to be tested. The second project involves a description of such agents: bispecific antibodies that simultaneously bind to an immune effector cell and a tumor cell. The bispecific antibody described here consists of folate attached to anti-T cell receptor antibodies, or their fragments. A single-chain Fv coupled with folate can redirect the lysis of human tumor cells that bear the high affinity folate receptor. Preliminary *in vivo* data showed that the folate/antibody conjugates were also capable of mediating rejection of the human tumor. This transgenic mouse model should now allow the evaluation and optimization of bispecific agents that can redirect a patient's own T cell response.

Keywords: Cytotoxic T Lymphocytes/Transgenic Mice/Bispecific Antibodies/Folate Receptor

Introduction

Most antibody-mediated targeting systems rely on the delivery of a linked, toxic compound in order to destroy the tumor. These approaches must overcome potential side effects associated with the toxic compound, either in the conjugate form or after release from the antibody. An alternative strategy, that has now been under investigation for the past 12 years, is to use the patients own T cells as the "toxic" moiety. There are several potential advantages of using T cells in this capacity: 1) cytotoxic T lymphocytes (CTL) are present in large numbers in an individual; 2) each CTL has multiple mechanisms for killing a target cell, thereby limiting the outgrowth of tumor variants that might escape a single mechanism; 3) each CTL can kill multiple tumor targets; and 4) CTLs are a normal (i.e. self) component of an individual, and thus they are not recognized and cleared by the immune sytem, as might be the case with some toxins.

One strategy to redirect the activity of T cells to a tumor has been the use of bispecific antibodies [1] that contain one antibody to a tumor antigen and another antibody to the T cell receptor complex (TCR). The bispecific antibody can mediate the lysis of the tumor cell by bridging CTL and tumor cell and by activating the CTL lytic machinery through the TCR complex. However, before a CTL can kill a tumor cell, it must be activated through simultaneous triggering of multiple cell surface molecules. There are now various strategies to accomplish such activation [2]. The goals of our work are to understand how a patients own CTLs might be most easily and effectively used, in conjunction with bispecific antibodies, as a therapeutic agent in cancer.

Two projects will be described in this report. The first involves the generation of a transgenic strain of mice (TCR/RAG) that will accept human tumor transplants, yet which contains endogenous CTLs. Thus, various drugs that can potentially be used for the activation and retargeting of CTLs to human tumors *in vivo* can be tested in these mice. Examples of the potency of tumor rejection by CTLs in these TCR/RAG mice will be shown. The second project involves the design and evaluation of the smallest bispecific antibodies yet produced, a conjugate of folate and an anti-TCR single-chain antibody (~ 30 kDa). These conjugates target the high affinity folate receptor (FR) present on most ovarian cancers and some brain tumors [3, 4].

Materials and Methods

Generation of Transgenic Mice. Mice that express αβ TCR trangenes from the CTL clone called 2C [5, 6] were crossed with recombination activating gene-1 knockout mice (RAG-/-)[7]. Some T cells from the TCR transgenic mice express endogenous TCR and thus these mice contain T helper cells and they are capable of rejecting allogeneic and xenogeneic tumor transplants. In contrast, RAG-/- mice do not contain either B cells or T cells because they are not able to rearrange either Ig or TCR genes. The progeny of this cross and F1 backcrossed mice were examined for serum IgM and with the 2C TCR-specific mAb 1B2 [8] in order to develop a colony of TCR/RAG-/- mice. Serum IgM was monitored with an enzyme-linked immunoassay in which anti-kappa IgG coated on plates was used to capture serum Ig and bound IgM was detected with an HRP-labeled anti-mouse μ secondary antibody. To control for the total amount of serum added, various serum dilutions containing lysed red blood cells were analysed for hemaglobin that was released, at an absorbance of 410 nm.

Tumor Cell Lines and Transplantation. Tumor cell lines were used either as targets in cytotoxicity assays or in transplantation experiments with TCR/RAG mice. The following DBA/2 (H-2^d) derived tumor cell lines [9, 10] were used as *in vitro* target cells: Mel, a murine erythroleukemia cell and F2-MTX^{r}A, a methotrexate resistant line selected for increased expression of FR by growth on low folic acid. The allogeneic mastocytoma line P815 (H-2^d), the syngeneic lymphoma EL-4 (H-2^b) and the human tumor cell lines SKOV-3, SKBR-3, BT474 (each erbB-2^+), and the FR$^+$ tumor KB [11] were transplanted into TCR/RAG mice and, in some cases, used as target cells *in vitro*.

Tumor cells, generally 10^7 for intraperitoneal experiments and 10^6 for subcutaneous experiments, were washed thoroughly in PBS and injected into either the peritoneal cavity or the shaved back of mice. For subcutaneous tumors, growth was assessed weekly or biweekly using calipers. Mean tumor diameter was calculated as the average of two perpendicular measurements. Animals were sacrificed when tumors reached diameters above 25 mm, or if the mice became overtly ill or the tumor became ulcerated. Mice injected i.p. were examined daily. Survival was assessed as

either the day of death or the point when moribund mice were sacrificed. Observation of mice injected i.p. was continued through a ten week period.

Bispecific Antibodies. The following monoclonal antibodies were used: 1B2, a mouse IgG1 specific for the TCR of CTL 2C [8] and KJ16, a rat IgG antibody specific for the Vβ8 region of the TCR [12]. KJ16 Fab fragments and KJ16 scFv were generated and purified as described previously [13]. Briefly, scFv was refolded from inclusion bodies and monomeric scFv was purified by G-200 HPLC purification. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously [14, 15]. Antibody conjugates were analyzed by mass spectrometry and absorption spectrophotometry.

In Vitro CTL Assays. The activity of CTL were examined in a cytotoxicity assay, in which tumor target cells were labeled with ^{51}Cr and effector cells were added with or without bispecific antibodies. Folate/antibody conjugates and cells were diluted in folate-free media and added to triplicate wells. Plates were incubated for 4 hours and supernatants were removed for gamma counting. Cytotoxicity was determined by: % specific release = ({experimental counts- spontaneous counts}/{maximal counts - spontaneous counts} x 100), where spontaneous release is measured in the absence of CTLs and maximal release is measured with detergent lysis.

Results and Discussion

Results of the two projects involving tumor cell targeting with bispecific antibodies and T cells will be described. The first project is designed to evaluate the potency of CTL-mediated tumor rejection, by using a transgenic mice that express CTLs but not T-helper cells or B cells. The second project describes an approach to redirecting the activity of CTL, using a novel form of bispecific antibody.

Transgenic Mouse Model for CTL-Mediated Tumor Rejection. In the normal host response to tumor antigens, there is a complex interaction among immune cells that leads to the elimination of the tumor cell. This interaction is known to involve T helper cells, antigen presenting cells (e.g. B cells and macrophages), and CTLs. We wished to determine if CTLs alone are capable of eliminating tumor cells and, if so, what strategies might be optimum for redirecting their activity exclusively to a tumor burden. Furthermore, we wished to develop an animal model that would allow human tumors to be successfully transplated but would also contain endogenous CTL for *in vivo* targeting studies (unlike nude and SCID mice which lack T cells).

To approach these questions, a strain of mice (TCR/RAG) was produced that contains only CTLs of a single TCR type and specificity (and which does not recognize any known human tumor antigens)(2)(Figure 1). The TCR/RAG mice were produced by breeding a transgenic mouse that expresses the α and β chains from CTL clone 2C [5, 6] and RAG-1 knockout mice [7]. The phenotype of adult TCR/RAG animals was examined in order to determine if they indeed lacked all B cells and T helper cells (i.e. CD4+ cells). Screening of offspring of different ages, from matings of TCR tg mice with RAG -/- mice, was performed by flow cytometry of spleen cells. These results revealed that essentially all of the splenic T cells are $CD8^+$ (i.e. CTLs)(Table 1) and that they all express the same TCR as identified with an anti-TCR antibody, 1B2 [8]. TCR/RAG mice exhibited, on average, slightly reduced spleen and thymus sizes conpared to normal mice or TCR transgenic mice (Table 1 and data not shown). The mice also lack any B cells, as evidenced by the lack of serum IgM (Figure 2).

Despite the presence of only CTLs in the TCR/RAG mice, CTLs could be activated both *in vitro* and *in vivo* with appropriate stimulating antigens. The transgenic TCR recognizes a peptide p2C (LSPFPFDL) bound to the class I MHC L^d [16] or a peptide SIYRYYGL bound to the class I MHC K^b [17]. Spleen cells from TCR/RAG mice injected with the SIYRYYGL peptide or spleen cells that were cultured with either SIYRYYGL/K^b or p2C/L^d proliferated and were shown to be activated by various criteria: expression of IL-2 receptors, expression of the CD69 antigen, and their ability to kill tumor cells that bear these antigens (Manning et al. submitted for publication).

In addition, the TCR/RAG mice were fully capable of eliminating tumor cells that bear the appropriate peptide/MHC ligand recognized by the tg TCR. The p2C/L^d complex is expressed by the DBA/2 tumor P815, but not by various other tumor cell lines. To investigate whether the TCR/RAG could reject this allogeneic tumor load, P815 cells were injected either subcutaneously or intraperitoneally. The TCR/RAG animals injected with 10^7 cells showed no signs of illness and with one exception, survived without event (Figure 3). Control RAG-/- animals failed to reject the allogeneic tumors and died or became moribund within three weeks. To examine the strength of the response, mice were injected with a 30 fold greater tumor load (3 x 10^8 P815 cells). Two of three high tumor load animals survived through the six week experimental period without event, and one succumbed to its tumor late in the course at day 42. The observation that tumors are rejected by the TCR/RAG animals and not rejected in the RAG mice indicates that the transgenic $CD8^+$ CTL is the cell type responsible for the rejection. In addition, TCR/RAG mice failed to reject other mouse tumors such as EL4, that do not express the appropriate ligand recognized by the transgenic CTL (Figure 4). This result further shows that peptide/L^d-specific CTL of the TCR/RAG mice are required for rejection.

Finally, various human tumor cell lines were transplanted into TCR/RAG mice in order to determine if they developed tumors and thus could be used as models for drugs that target CTLs to tumors. The erbB-2^+ tumors SKOV3 and BT474 (but not SKBR-3) and the high affinity FR^+ tumor line KB all grew as subcutaneous tumors (Figure 4 and data not shown). Thus, the KB tumor line can serve as a model for CTL targeting using folate/anti-TCR conjugates described below.

Characterization of Bispecific Folate/Antibody Conjugates. Bispecific antibodies that bind to a tumor antigen and the TCR redirect CTLs to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the FR, is expressed on most ovarian carcinomas. Recently, we showed that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein [18].

The size of these conjugates can be reduced to the smallest bispecific agent yet described (30-kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody called KJ16 (Figure 5). Folate was attached through a carboxyl group to scFv amines using a carbodiimide reaction. The scFv/folate conjugates were as effective as intact IgG/folate conjugates in mediating lysis of FR^+ tumor cells by CTL (Figure 6)[19]. The optimal folate density was in the range of 5 to 15 folate molecules per scFv or IgG molecule, which yielded EC_{50} values of approximately 40 pM (1.2 ng/ml for scFv). The scFv/folate conjugates could also efficiently target tumor cells *in vitro* even in the presence of free folic acid at concentrations that are normally found in serum [19].

In vitro-activated CTLs from the TCR/RAG mice described above were able to kill the human FR+ tumor line KB in the presence of folate/scFv and folate/Fab conjugates (Figure 7). This redirected lysis was completely inhibited by excess free folate and thus the effect is mediated through the high affinity FR.

Finally, a preliminary study has shown that a Fab/folate conjugate was capable of mediating the rejection of KB tumor cells in TCR/RAG mice that had been injected with SIYRYYGL peptide to activate CTL (Figure 8). In this experiment, mice were co-injected with 3 million KB cells and 20 μg of the folate/Fab conjugate subcutaneously. Studies to further evaluate the *in vivo* targeting efficiency of the folate/scFv and folate/Fab conjugates will focus on several issues: 1) methods of *in vivo* CTL-activation; 2) route and timing of antibody delivery; and 3) optimization of the effective concentration and forms of the conjugates.

Conclusion

Using a transgenic animal model, we have demonstrated that CTL alone are potent mediators of tumor rejection. This finding suggests that it will be possible to focus strategies on the *in vivo* activation and retargeting of CTL to specific tumor cell antigens. In addition, the TCR/RAG mice can accept human tumor xenografts and thus they should serve as a useful model for the testing of bispecific agents that target human tunors.

In an effort to generate smaller and more potent bispecific agents, we have explored the use of folate/anti-TCR conjugates for targeting the high affinity FR, a tumor associated antigen present on most ovarian tumors. Folate directly coupled to a single chain V_LV_H antibody yielded a potent tumor targeting agent in cytotoxicity assays with CTLs. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors, in reduced immunogenicity, and in eliminating Fc-mediated side effects.

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Figure legends

Figure 1. Features of the 2C CTL TCR transgenic mice, RAG-1 knockout mice, and the TCR/RAG$^{-/-}$ progeny.

Figure 2. ELISA of serum from a normal C57Bl/6 mouse, a RAG-1$^{-/-}$ mouse, and a TCR/RAG$^{-/-}$ mouse. Blood was lysed by hypotonic shock and diluted for analysis in 96-well plates. Absorbance of the diluted blood was monitored at 410 nm prior to completion of the ELISA. ELISAs for serum IgM were performed as described in Materials and Methods.

Figure 3. Tumor allograft rejection in TCR/RAG mice. In the panel on the left, TCR/RAG$^{-/-}$ mice were injected i.p. with either 10^7 P815 cells (n=5 mice) or 3 x 10^8 P815 cells (n=3 mice). RAG-1$^{-/-}$ mice received injections of 10^7 P815 cells (n=5 mice). Animals were monitored as described in *Materials and Methods* for a period of ten weeks. In the panel on the right, RAG-1$^{-/-}$ or TCR/RAG$^{-/-}$ mice (n=3) were injected s.c. in the shaved back with 10^6 P815 cells in 100 µl and monitored weekly for tumor growth. Mean tumor diameter represents the average of two perpendicular measurements.

Figure 4. TCR/RAG$^{-/-}$ mice do not reject tumors that lack the appropriate peptide/MHC ligand. In the panel on the left, TCR/RAG mice were injected s.c. with 10^6 EL4 (H-2^b) or P815 (H-2^d) in the shaved back in 100 µl of PBS. In the panel on the right, TCR/RAG$^{-/-}$ mice were injected s.c. with 3 x 10^6 human KB tumor cells. Mice received either 10 nmoles peptide SIYRYYGL (+SIYRYYGL) or saline (- SIYRYYGL) 2 days prior to tumor transplantation. N = 3 for all experiments. The absence of error bars indicates a standard deviation smaller than the size of the symbol.

Figure 5. Diagram of the single-chain Fv from the anti-T cell receptor antibody KJ16. The 26 amino acid linker that contains multiple lysine residues for carbodiimide coupling of folate is shown.

Figure 6. Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells. Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 μM EDC, 100:1 molar ratio of folate:antibody) yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C.

Figure 7. Folate/antibody conjugates mediate lysis of FR^{+} human tumor cell line KB by CTLs. Activated CTL from TCR/RAG mice were added to ^{51}Cr-labeled KB cells in the presence of the indicated agents. Fab/folate and scFv/folate were each assayed at ~ 10 μg/ml. Free folate was added at 1 μM.

Figure 8. Fab/folate conjugates mediate the rejection of KB tumor cells in TCR/RAG mice. TCR/RAG mice were injected s.c. with 3 x 10^{6} human KB tumor cells in the presence (+) or absence (-) of 20 μg Fab KJ16/folate. Mice received 10 nmoles peptide SIYRYYGL 2 days prior to tumor transplantation. N = 3 for all experiments. The absence of error bars indicates a standard deviation smaller than the size of the symbol.

Table 1. Flow cytometric analysis of splenic lymphocyte populations in TCR/RAG-1$^{-/-}$ mice.

Strain/Age	Sex	Percent of T cells*			Spleen wt (mg)	Cell count ($x10^7$)
		CD4+8-	CD4+8+	CD4-8+		
C57/B6						
5 mo	F	66	0	33	80	5
5 mo	F	55	0	45	82	6
5 mo	M	63	0	37	228	
2C TCR						
5 mo	F	31	0	72	107	
TCRRAG-/-						
1 mo	F	1	1	98	36	.6
1 mo	M	4	2	94	80	1.7
2 mo	F	1	1	98	92	3.2
2 mo	F	1	1	98	54	2.2
2 mo	M	2	1	97	52	1.1
2 mo	M	1	1	98	42	
3 mo	M	1	1	98	113	
3 mo	M	5	3	93	98	
4 mo	M	2	0	98	47	1.9
4 mo	M	1	0	99		
5 mo	M	1	2	96	75	
5 mo	M	1	0	99	144	
5 mo	F	0	2	98	91	
5 mo	F	0	1	99	58	
6 mo	M	5	0	95	48	
6 mo	F	1	0	99	186	
TCRRAG-/- median		1	1	98	75	

*Total T cells was calculated as (CD4+8-)+(CD4+8+)+(CD4-8+).

Figure 1 - Kranz et al.

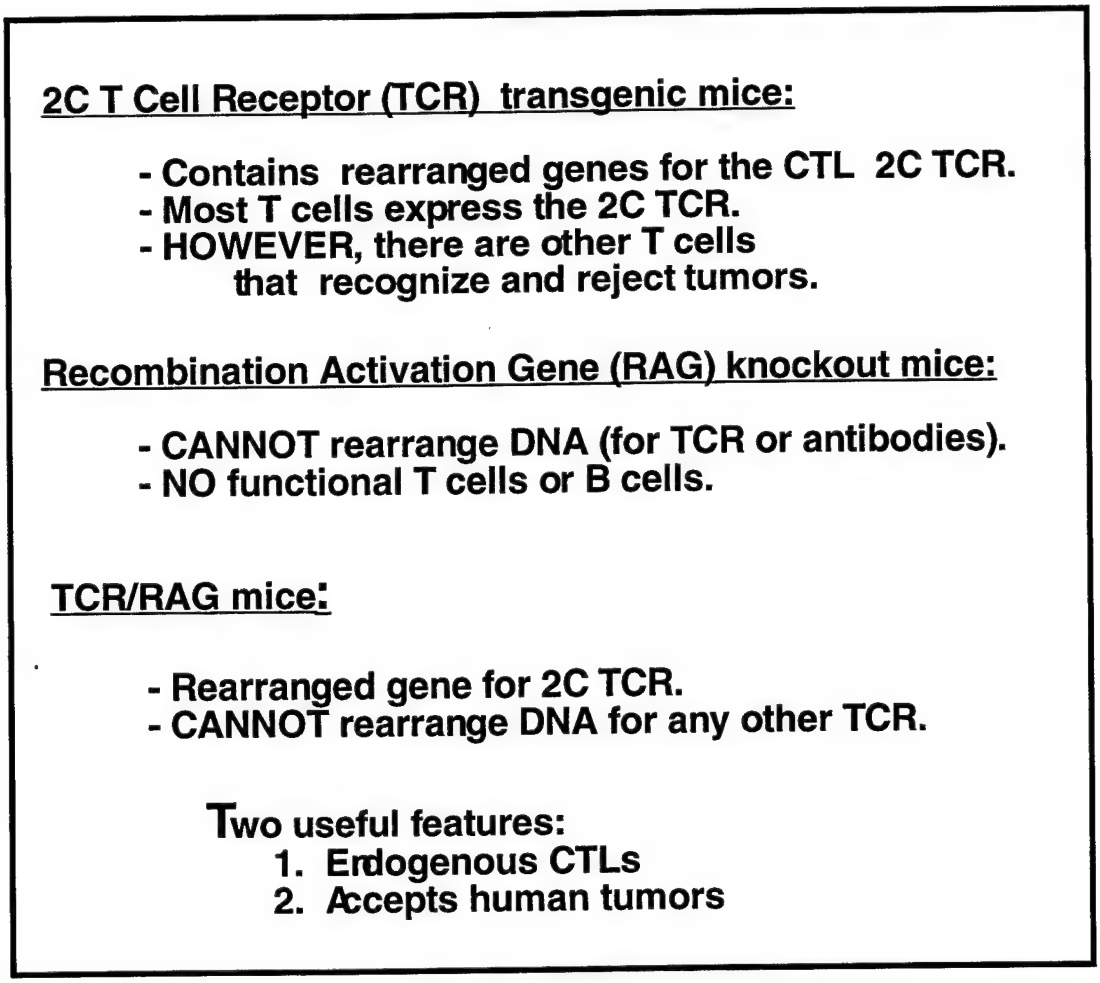


Figure 2 - Kranz et al.

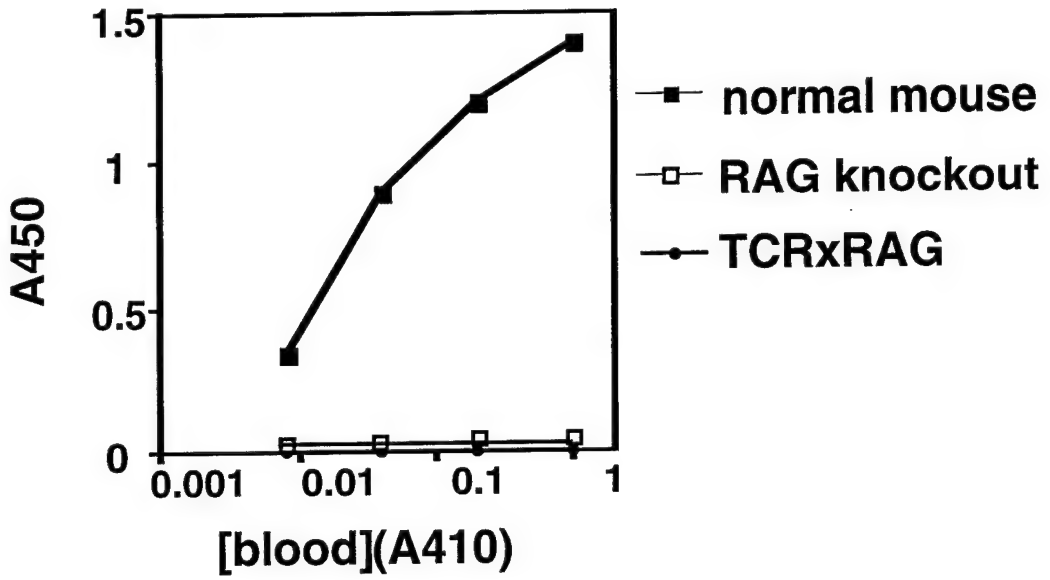
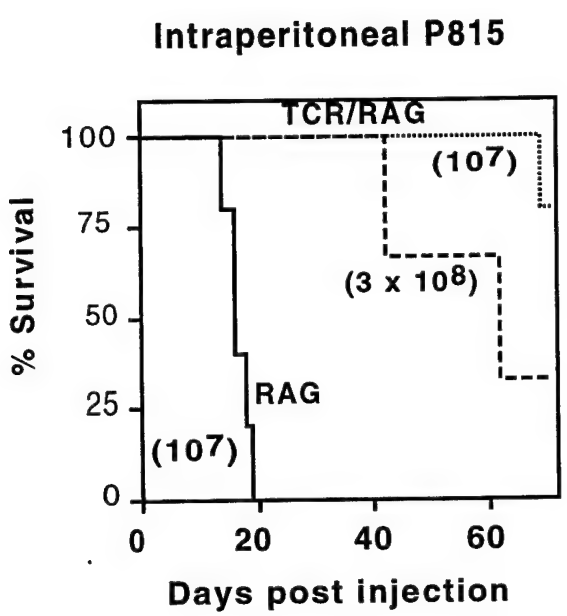


Figure 3 - Kranz et al.



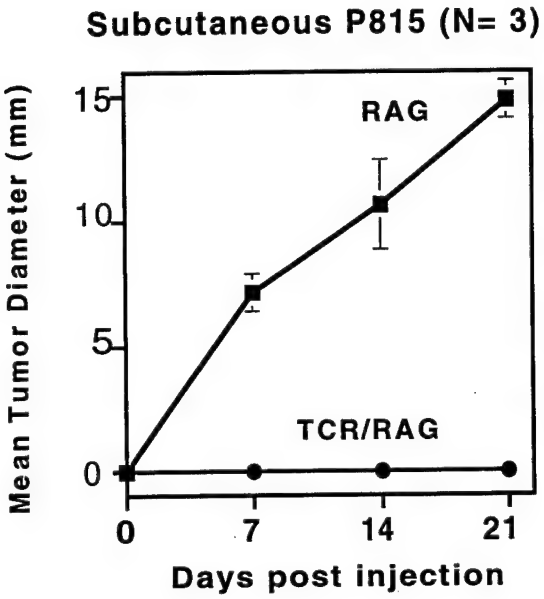


Figure 4 - Kranz et al.

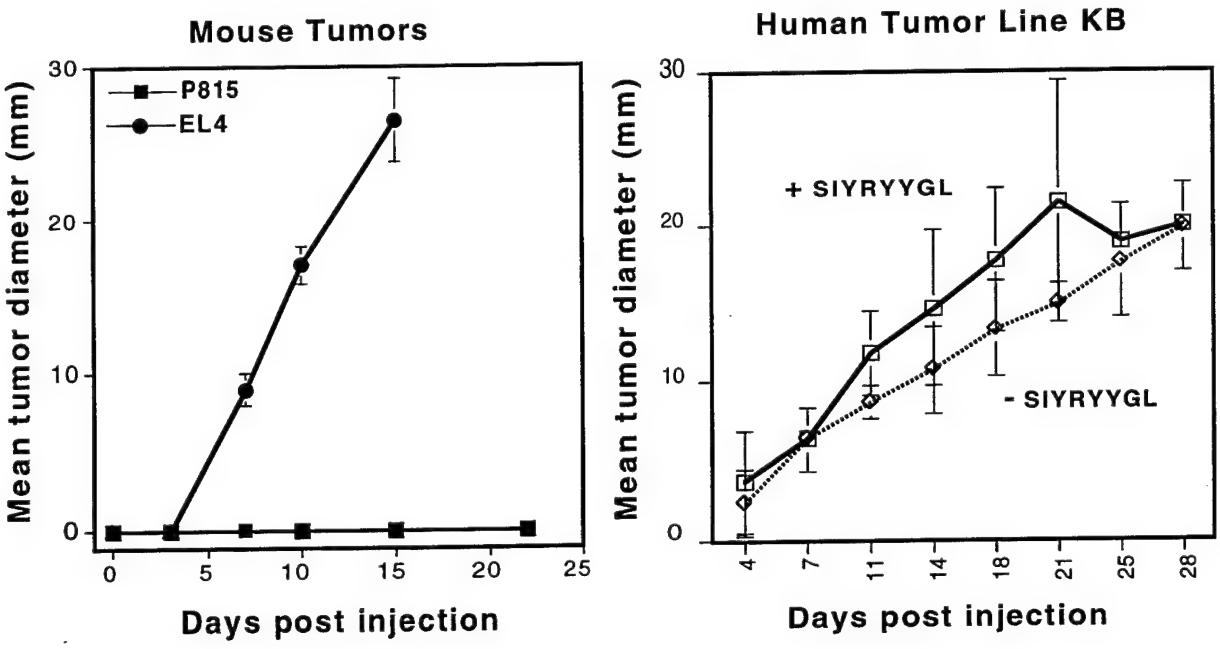
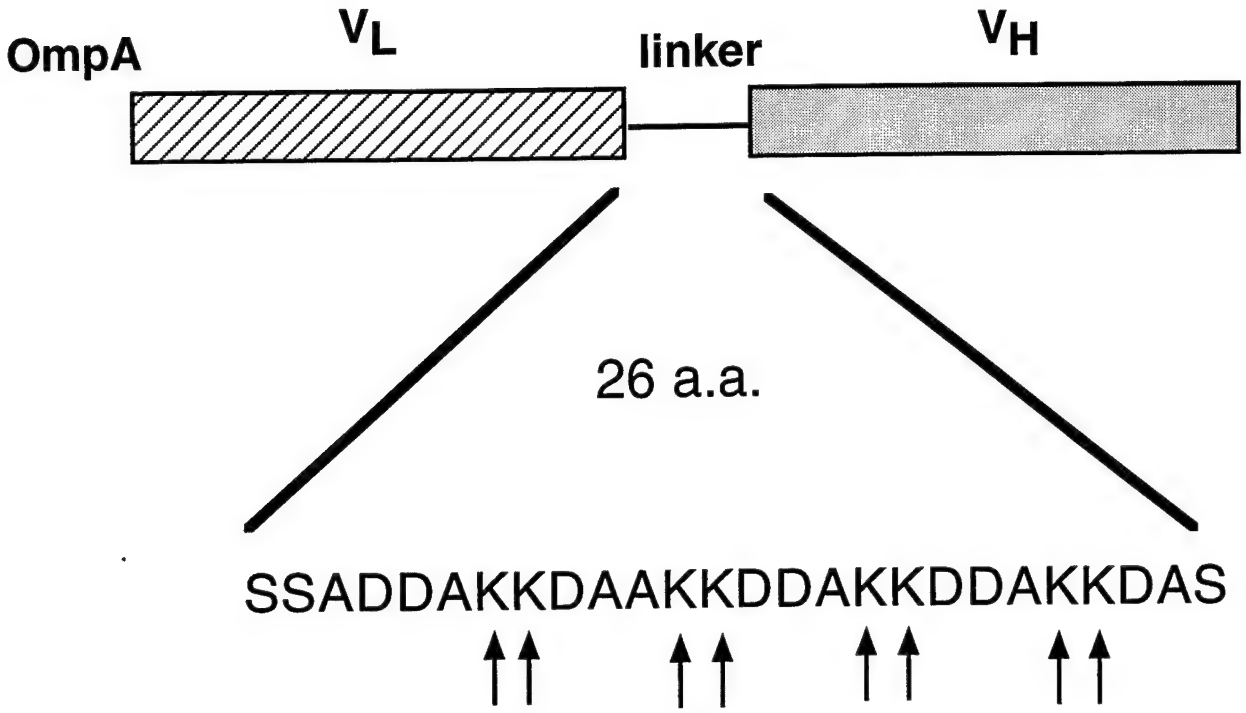


Figure 5 - Kranz et al.



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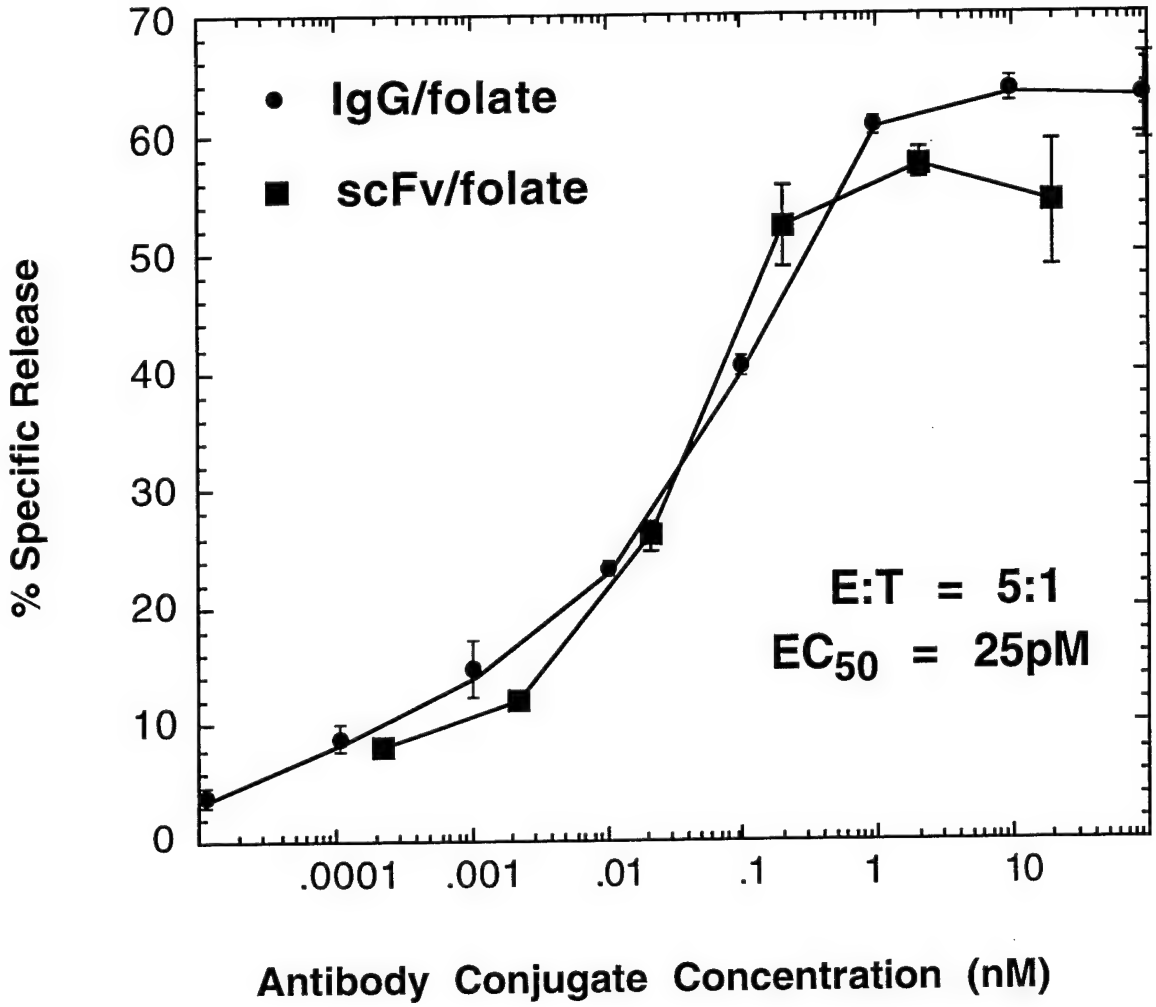


Figure 7 - Kranz et al.

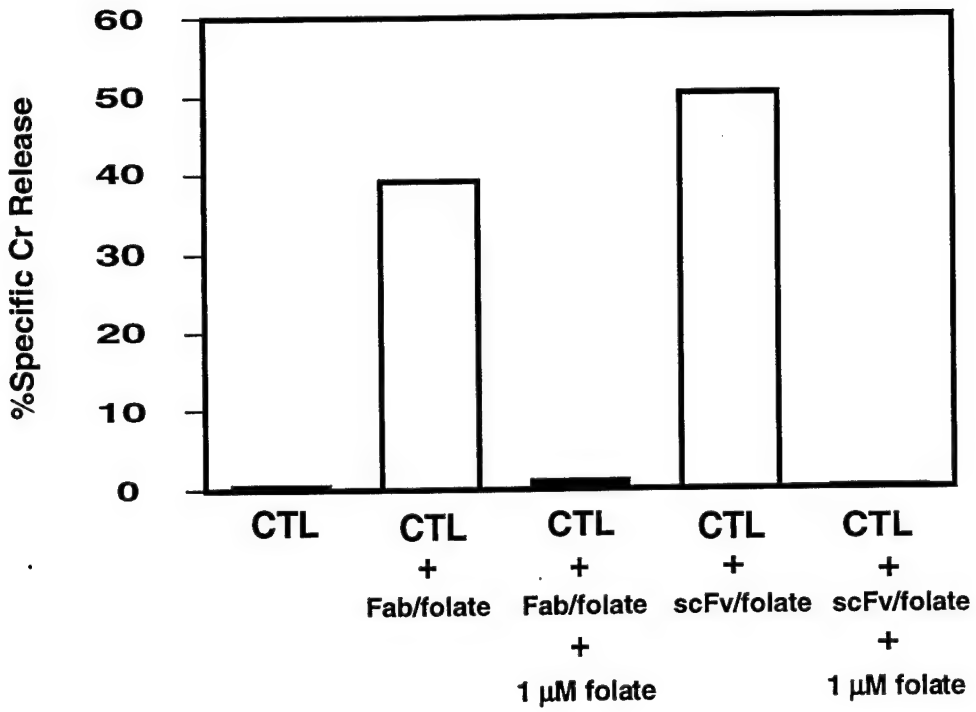
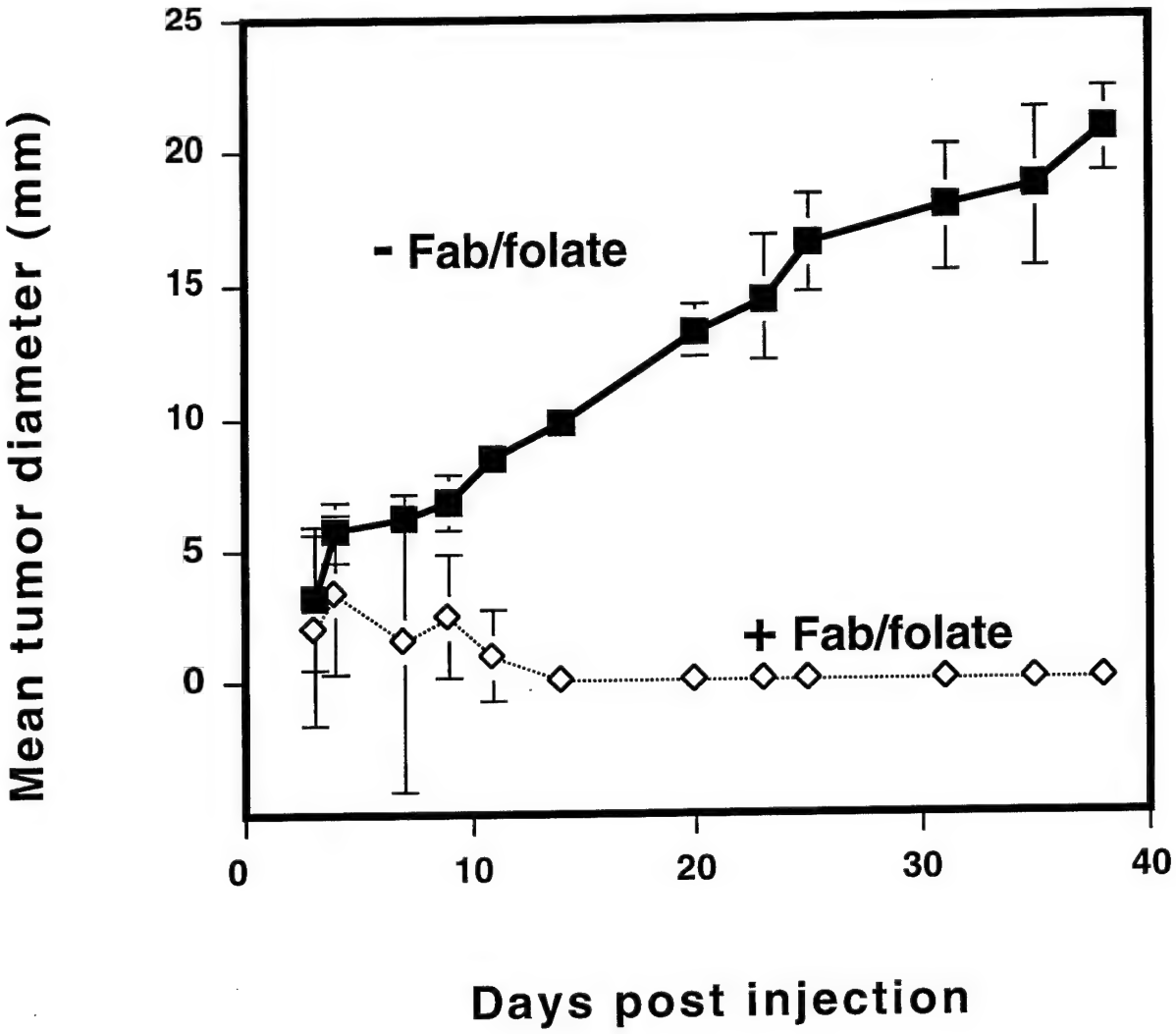


Figure 8 - Kranz et al.



Antigen Recognition and Allogeneic Tumor Rejection in CD8$^+$ TCR Transgenic/RAG$^{-/-}$ Mice[1]

Thomas C. Manning,* Laurie A. Rund,* Meegan M. Gruber,* Francesca Fallarino,† Thomas F. Gajewski,† and David M. Kranz[2]*

Three sources of help for the development of a CD8$^+$ CTL response have been described: the CD4$^+$ direct and indirect pathways and the CD8$^+$ direct pathway. In an effort to understand the minimal requirements for the development of a CTL response in vivo, we have bred mice transgenic for the 2C TCR onto a RAG$^{-/-}$ background. The 2C T cells in this animal are exclusively CD8$^+$ CTLs of a single specificity, and they exhibit altered thymic maturation compared with that of T cells from 2C TCR/RAG$^{+/+}$ mice. T cells from 2C TCR/RAG$^{-/-}$ mice can be activated to a high level in vivo by administration of a self-MHC-restricted antigenic peptide. The 2C TCR/RAG$^{-/-}$ mice are able to reject B7-negative allogeneic tumors bearing the appropriate peptide/MHC ligand p2C/L^d. These mice fail to reject syngeneic tumors, and their RAG$^{-/-}$ littermates lacking 2C T cells uniformly succumb to both allogeneic and syngeneic tumors. Moreover, blockade of B7 costimulatory molecules fails to prevent tumor rejection in the 2C TCR/RAG$^{-/-}$ mice, suggesting that allorejection is occurring independently of B7-mediated costimulation as well as in the absence of CD4$^+$ T cells. CTLs isolated from the site of the tumor during the period of rejection express the activation marker CD25 and are able to mediate ex vivo cytolysis of tumor cells bearing the appropriate Ag. These results suggest that in this TCR transgenic model with a very high precursor frequency, CTL development can occur in the absence of B7:CD28 costimulation and without CD4$^+$ help. ***The Journal of Immunology,*** **1997, 159: 0000–0000.**

Transplantation of tissue containing mismatched MHC Ags generally produces a vigorous alloreactive response. The strength of such a response has been attributed to a high frequency of T cells (~10%) capable of recognizing alloantigen. While pathogen-specific T cells generally recognize a foreign peptide in the context of self-MHC, alloreactive T cells usually recognize self peptides in the context of a foreign MHC molecule. Three possible sources of help have been described in the generation of an alloreactive CTL response: 1) the direct CD4$^+$ pathway, 2) the indirect CD4$^+$ pathway, and 3) a direct CD8$^+$ pathway (1). Where it has been examined in tumor models, an additional CD8$^+$ indirect pathway is predominant in CTL development (2, 3). The direct CD4$^+$ pathway involves Th cells that recognize antigenic peptide/MHC-II complexes on donor cells. The indirect CD4$^+$ pathway involves Th cells that recognize peptides derived from donor class I MHC presented by self-MHC class II molecules on self-APCs (4). The direct CD8$^+$ pathway involves CTL recognition of peptide/MHC class I complexes on donor cells and is considered to be highly dependent on the number of donor professional APCs in a graft (5). The CD8$^+$ indirect pathway consists of initial CTL cross-priming by host bone marrow-derived APCs and requires the uptake and presentation of tumor Ags on host MHC class I molecules (2, 3). The relative importance of the various sources of help for CTL activation is not fully understood, but the dominant pathway probably depends on the scenario encountered. Studies using CD4$^-$CD8$^-$ double knockout mice showed that such mice could generate alloreactive T cells and that these animals were able to reject allogeneic skin grafts (6). Despite this finding, a recent report using separate CD4$^-$ and CD8$^-$ knockout mice has stressed an absolute requirement for CD4$^+$ cells in the initiation of allograft rejection (7).

It is well established that two signals are required for the induction of an optimal T cell response. TCR ligation of antigenic peptide/MHC complexes provides signal 1, while costimulation, most importantly through CD28 ligation by B7 molecules, provides signal 2 (8; reviewed in Refs. 9 and 10). Ligation of the TCR in the presence of costimulation leads to proliferation, while TCR ligation in the absence of costimulation produces either no response or a state of hyporesponsiveness. Recently, it has been demonstrated that CD28:B7 ligation serves to prolong cell survival and prevent apoptotic death by up-regulation of the Bcl-xL molecule (11). The B7 family of costimulatory molecules has been shown to be important in both the development of a T cell response and, more specifically, in allograft rejection. This has led to many efforts to prevent T cell activation using strategies that allow signal 1, but block signal 2. For example, B7 blockade with CTLA4Ig (12) has shown protection in several animal models of graft rejection (13–15). However, it appears that costimulation either by IL-2 or through CD28:B7 interactions is not an absolute requirement for the development of all T cell responses (16–19).

The 2C TCR is one of the most well-characterized TCRs. The CTL clone 2C was initially isolated from a BALB.B mouse as an allospecific T cell that recognized L^d on the mastocytoma P815 (20). The binding affinity of the 2C TCR for its defined alloantigen p2C/L^d has been measured (21), and the crystal structure of the receptor is now known (22). In addition to its primary Ag, peptide p2C bound to L^d, the 2C TCR also binds the Ags SIYRYYGL/K^b (23), dEV-8/K^b (24), and many of the Vβ8-specific superantigens

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(25). T cell development has been characterized in 2C TCR transgenic mice with the use of the clonotypic Ab 1B2 (26–28). We have now crossed 2C TCR mice onto a RAG-1$^{-/-}$ background (29) to produce animals with a uniform population of resting CTL precursor cells. These TCR/RAG$^{-/-}$ mice (hereafter referred to as TCR/RAG mice) contain 95% CD8$^+$1B2$^+$ peripheral T cells and lack B cells and T cells of any other specificity. This strain provides an opportunity to examine the requirements for CTL activation in a defined system with well-characterized Ags.

To investigate the ability of 2C TCR/RAG mice to recognize alloantigen in vivo, the DBA/2-derived P815 tumor line (30), against which CTL clone 2C was originally selected, was used in various transplantation experiments. The P815 model of tumor rejection has also been studied extensively, and recent work has used various transfectants of this cell line to evaluate the effect of the costimulation by B7 family molecules that are ligands for CTLA-4 and CD28 (31–33). Possible immune recognition and elimination of P815 by 2C TCR/RAG mice was of interest because this system would lack the three well-characterized sources of help for the development of an alloreactive CTL response; there are no CD4$^+$ Th cells for either direct or indirect help, and because P815 does not express B7, there is not a clear source of signal 2 for direct CTL stimulation. Despite these deficiencies, we show that large allogeneic tumor burdens are indeed rejected in a CD8-dependent manner in the absence of B7-mediated costimulation or help from CD4$^+$ T cells.

Materials and Methods

Mice

2C TCR transgenic mice (27) were crossed with RAG-1$^{-/-}$ mice (29) obtained from The Jackson Laboratory (Bar Harbor, ME). F_1 mice were backcrossed to RAG$^{-/-}$ to yield TCR/RAG$^{-/-}$ mice. Mice were maintained in barrier cages at the University of Illinois animal care facility. BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IA). Transgenic mice used in experiments were between 6 and 12 wk of age. Additional experiments were performed using 2C TCR transgenic mice crossed onto a RAG-2$^{-/-}$ (34) background. These animals as well as DBA/2 mice were bred and housed at the University of Chicago animal care facility.

Cell lines and Abs

P815 is the mastocytoma-derived tumor line (30) against which T cell clone 2C was initially selected (20). EL-4 is a syngeneic (H-2^b) T lymphoma-derived cell line. T2-L^d is a human lymphoblastoid derived line deficient in peptide transport and transfected with the L^d gene (35). All cell lines were maintained in complete RPMI 1640 medium containing 5 mM HEPES, 10% FCS, 1.3 mM L-glutamine, 50 μM 2-ME, penicillin, and streptomycin. 1B2 is a mouse IgG1 mAb that is clonotypic for the 2C TCR. The Abs 53-6.7 (anti-CD8), RM4-5 (anti-CD4), 53.2.1 (anti-Thy-1.2), H1.2F3 (anti-CD69), 7D4 (anti-CD25), and 4F10 (anti-CTLA-4) were obtained from PharMingen (San Diego, CA). Ab 30-5-7, a mouse IgG2a anti-L^d mAb, is sensitive for peptide-bound L^d molecules (36). P198 is an immunogenic tum$^-$ variant of P815 that was isolated and grown as previously described (37).

Peptides

The peptides SIYRYYGL (23), dEV-8 (24), MCMV, p2C (38), and QL9 (39) were synthesized on an Applied Biosystems 430A instrument (Foster City, CA) using standard F-moc chemistry at the University of Illinois Biotechnology Center (Urbana, IL) and were analyzed for purity by mass spectrometry and for concentration by quantitative amino acid analysis. Peptides were purified by reverse phase HPLC on a C_{18} column eluting with a linear 5 to 60% acetonitrile gradient over 60 min in 0.1% trifluoroacetic acid.

Proliferation assays

Standard proliferation assays were performed in triplicate round-bottom wells of 96-well plates over a 72-h period in complete RPMI medium. [^{3}H]thymidine at 1 μCi/well was added during the last 18 h of the incubation period. Cells were collected and harvested on glass filters using a semiautomated cell harvester (Cambridge Technology, Inc., Watertown, MA). In some experiments, supernatant from rat splenocytes treated with Con A was added at a 10% volume to the assays. Con A supernatant was combined 2/1 with 20% α-methylmanoside to neutralize free Con A.

Cytotoxicity assays

Cytotoxicity was examined in standard ^{51}Cr release assays. Briefly, target cells were labeled with ^{51}Cr for 1 h at 37°C. Some 10^4 target cells/well were incubated with various numbers of effector cells for 4 h. Supernatants were harvested and assayed for specific lysis according to the formula: percent specific lysis = (counts per minute experimental − counts per minute spontaneous)/(counts per minute maximal − counts per minute spontaneous). In all cases spontaneous lysis was <25% of maximal lysis.

Tumorigenicity experiments

Tumor cells, generally 10^7 for i.p. experiments and 10^6 for s.c. experiments, were washed thoroughly in PBS and injected into either the peritoneal cavity or the left flank of mice. For s.c. tumors, growth was assessed weekly or biweekly using calipers. Mean tumor diameter was calculated as the average of two perpendicular measurements. Animals were killed when tumors reached diameters >25 mm, or if the mice became overtly ill or the tumor became ulcerated. Mice injected i.p. were examined daily. Survival was assessed as either the day of death or the point when moribund mice were killed. Observation of mice injected i.p. was continued through a 10-wk period.

Flow cytometry

Flow cytometry was performed at the University of Illinois Flow Cytometry Facility using a Coulter Epics XL instrument. Thymi or spleens were removed from mice and passed through wire mesh, and RBC were lysed. Generally, ~10^5 cells/tube were incubated for 30 min with the appropriately labeled Ab in a volume of 50 μl PBS/1.0% BSA on ice. Cells were washed and resuspended in 400 μl before analysis.

Treatment of mice with murine CTLA4IgG3 fusion protein (mCTLA4γ3)[3]

Purified mCTLA4γ3 (32) and control IgG3 were adjusted to 100 μg/ml in PBS. Mice received 1 ml of either of these reagents on days −1, 0, 1, 2, 4, 7, 11, 14, 17, 21, and 24. Tumor cells were implanted several hours following the second injection on day 0.

Results

Characterization of TCR/RAG mice

The progeny of TCR/RAG$^{-/-}$ F_1 × RAG$^{-/-}$ mice were examined for serum IgM levels and 1B2$^+$ PBL. Mice that exhibited no detectable IgM by ELISA or 1B2$^+$ cells by flow cytometry served as founders for a colony of 2C TCR/RAG mice (data not shown). Two-color flow cytometry analysis using anti-Thy 1.2, and the clonotypic Ab 1B2 revealed that essentially 100% (99.4 ± 0.7%) of the T cells from the spleens of these mice were 2C T cells (Fig. 1). This showed not only that the mice express the transgenic TCR, but it confirmed the RAG$^{-/-}$ background of these mice, as no endogenously rearranged TCR genes (i.e., Thy 1.2$^+$/1B2$^-$ cells) were observed. This contrasts with 2C TCR transgenic mice (hereafter referred to as TCR mice), in which only 88 ± 5% of the splenic T cells were 1B2$^+$ ($p < 0.001$).

T cells from spleens and thymi of mice at different ages were examined using 1B2 and anti-CD4 and anti-CD8 Abs. Three-color flow cytometric analysis of 2C T cells from the thymus revealed that TCR/RAG mice had more CD4$^+$/CD8$^+$ cells and fewer CD4$^-$/CD8$^-$ than did the TCR mice (Fig. 2A). Within the TCR/RAG thymus, 35 ± 12% of the 2C thymocytes were CD4$^+$CD8$^+$, while in the TCR thymus, only 19 ± 7% of the 2C thymocytes were CD4$^+$CD8$^+$ ($p < 0.01$). Conversely, the number of CD4$^-$CD8$^-$ cells was reduced (24 ± 9%) in TCR/RAG mice compared with that in TCR mice (41 ± 9%; $p < 0.005$). Thus,

[3] Abbreviations used in this paper: mCTLA4γ3, murine CTLA4IgG3 fusion protein; low, low level; PEC, peritoneal exudative cell.

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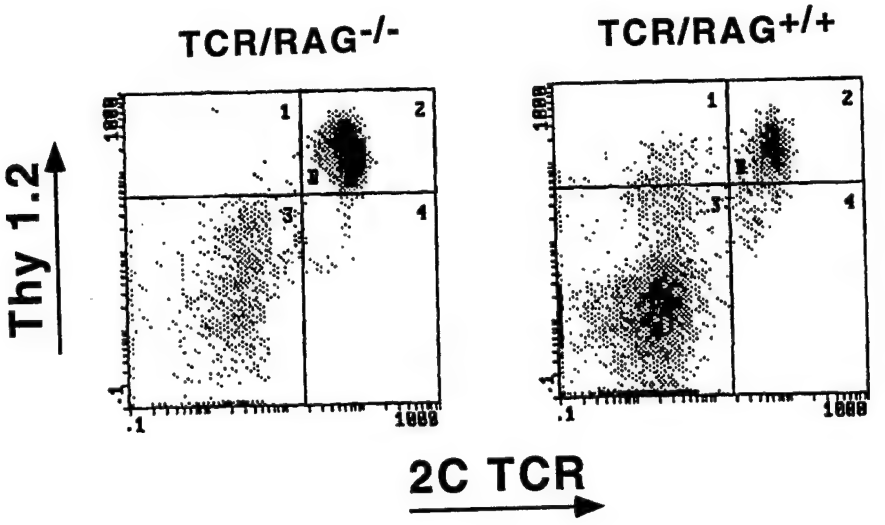

FIGURE 1. Characterization of T cell populations in TCR/RAG and TCR mice. Flow cytometry was performed as described in *Materials and Methods* using the mAbs 53.2.1 (anti-Thy 1.2) and 1B2 (2C TCR clonotypic).

TCR/RAG mice had about twice as many double-positive thymocytes and about half as many double-negative thymocytes as TCR mice. The significant number of $CD4^-CD8^-$ 2C T cells present in the spleens of TCR mice was also absent in the spleens of TCR/RAG mice (Fig. 2*B*). Within the spleens of TCR/RAG mice, 95 ± 2% of 2C T cells were $CD8^+$, while in spleens of TCR mice, only 65 ± 10% of 2C T cells were $CD8^+$, with the majority of the remainder being $CD4^-CD8^{low}$ or $CD4^-/CD8^-$ ($p < 0.0001$). Thus, in the TCR/RAG mice, ~95% of the entire T cell repertoire consists of $CD8^+$ CTL precursors, while in the TCR mice, only about 55% (45–70%) of all T cells are 2C $CD8^+$ CTL precursors. These results indicate that the prevention of endogenous TCR gene rearrangement during thymic development in the 2C TCR/RAG mouse leads almost exclusively to a $CD8^+$ CTL phenotype.

Ability of TCR/RAG to reject allogeneic tumors

Since 2C TCR/RAG mice lack B cells or Th cells, they provide a unique opportunity to examine the requirements for the development of a CTL response. The allogeneic tumor line P815 lacks the B7 family of costimulatory molecules and thus provides a source of signal 1 (p2C/L^d-TCR ligation) in the absence of signal 2 (B7-CD28 ligation). To investigate whether TCR/RAG mice could reject an allogeneic tumor, P815 cells were injected either s.c. or i.p.

In the i.p. model, control $RAG^{-/-}$ animals ($n = 5$) injected with 10^7 cells failed to reject the allogeneic tumors and died or became moribund and were killed within 3 wk (Fig. 3*A*). TCR/RAG mice ($n = 5$) injected with 10^7 cells showed no signs of illness and survived the 10-wk period, with one exception (discussed later). Survival of TCR/RAG mice was significantly prolonged compared with that of $RAG^{-/-}$ mice ($p < 0.005$). To examine the strength of the response, mice were injected with a 30-fold greater tumor load (3×10^8 cells). Survival of these high tumor load animals ($n = 3$) was also prolonged compared with that of $RAG^{-/-}$ mice ($p < 0.05$), but only one of the animals survived through 10 wk without event. TCR/RAG mice injected with 10^7 cells appeared to survive longer than those receiving 3×10^8 cells, although this result was not statistically significant ($p = 0.10$). In the s.c. tumor model, $RAG^{-/-}$ mice injected with 10^6 P815 cells uniformly developed tumors that grew to a large size within 3 wk, while the TCR/RAG animals remained free of tumors and exhibited no illness throughout a 6-wk period (Fig. 3*B*). Tumor recurrence in these animals had not been observed through day 60 ($n = 3$). The fact that tumors were rejected by TCR/RAG mice but not by $RAG^{-/-}$ mice indicates that the transgenic $CD8^+$ CTL is necessary for the rejection.

To confirm the specificity of tumor rejection, the response of 2C TCR/RAG mice to s.c. injection of syngeneic EL4 tumor cells ($H\text{-}2^b$) was examined. EL4 tumors grew rapidly, while P815 tumors were uniformly rejected (Fig. 4). Coinjection of equivalent numbers of EL4 cells together with P815 cells delayed the growth of EL4 tumors by about 1 wk ($p < 0.0005$), but once established, growth rates for the EL4/P815 mix were similar to those for EL4 alone. Thus, while some bystander activity or peptide/MHC-independent killing may be responsible for slowing the initial growth of EL4, 2C CTLs are only able to cause the rejection of tumors bearing Ag of the correct specificity (i.e., p2C/L^d).

Tumor recrudescence is not attributable to tumor variant escape

Two of the high tumor dose i.p. animals and one of the lower tumor dose i.p. animals exhibited tumor reoccurrence during the course of the 10-wk observation period. Analysis of one of these animals revealed a large ascites tumor yielding over 4×10^8 cells. Microscopic examination of a Wright's stained smear of this tumor demonstrated two distinct populations of cells: a smaller cell type appearing identical with P815 and a larger mononuclear granulocyte (data not shown). After culture for several days, a single cell population became dominant, which appeared identical with cultured P815 by microscopic examination. Flow cytometric analysis of the peritoneal lavage demonstrated that the recovered cells consisted of both L^{d+} P815 tumor cells and a large number of activated peritoneal macrophages that were Thy 1.2^- and $CD25^+$ (Fig. 5*A*). Staining with the anti-L^d Ab 30-5-7 demonstrated that the levels of L^d were identical for the recovered tumor cells and cultured P815 (data not shown).

A CTL killing assay confirmed that both the recovered P815 tumor and cultured P815 were identical in their susceptibility to lysis by activated 2C CTLs (Fig. 5*B*). The responsiveness of the 2C T cells from these animals was not investigated, but it is possible that TCR/RAG mice have defects associated with memory responses, as has recently been observed in a TCR transgenic model of viral immunity (40). Thus, it appears that while tumor control is generally achieved and maintained, in at least some of the animals there is a failure to completely eradicate tumor cells, and late regrowth of tumor can occur. Moreover, this tumor recrudescence is not attributable to down-regulation of L^d by the tumor cells or resistance to CTL-mediated killing.

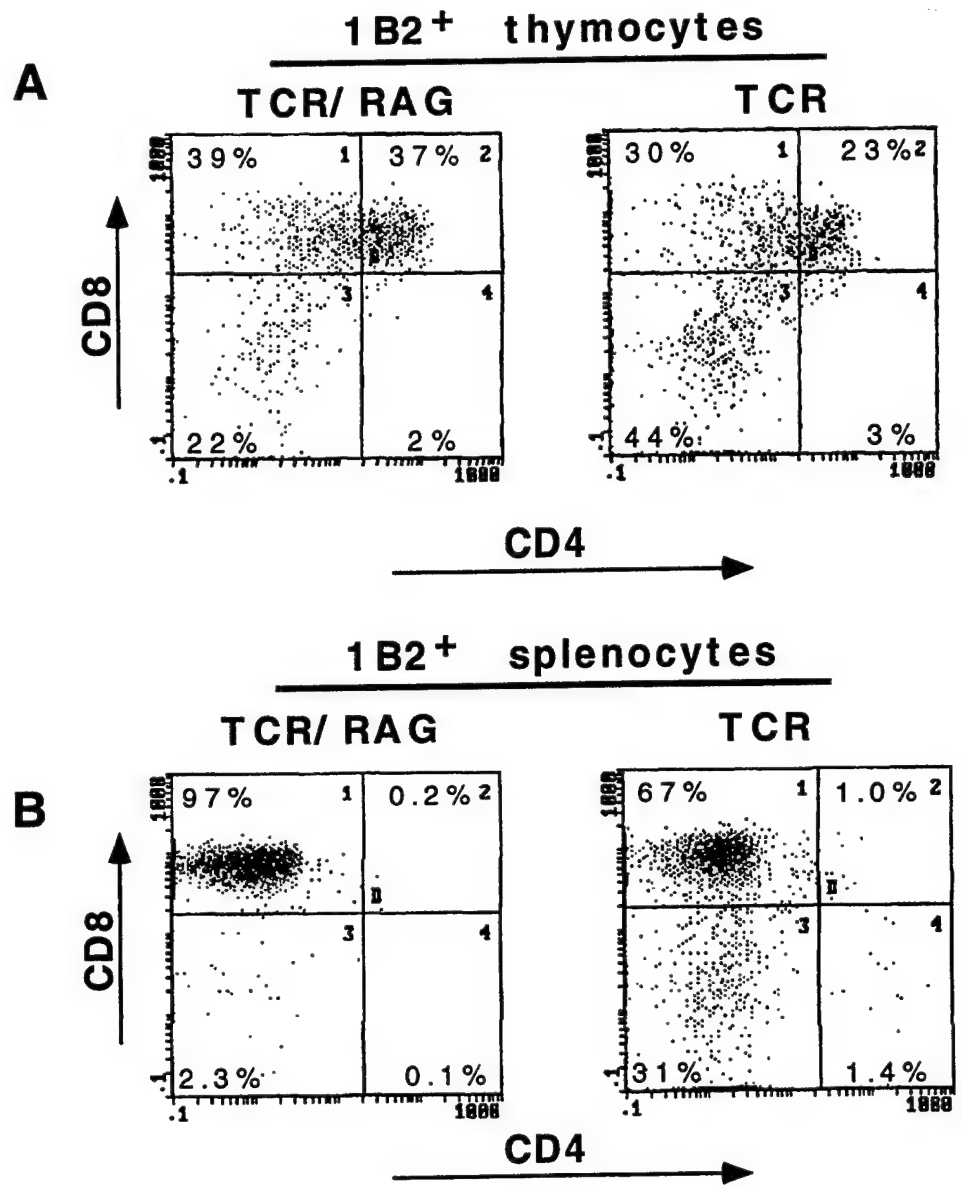

FIGURE 2. Comparison of 2C T cell populations from thymus and spleen of TCR/RAG and TCR mice. Flow cytometry was performed as described in *Materials and Methods* using the mAbs 1B2 (2C TCR clonotypic), 53-6.7 (anti-CD8), and RM4-5 (anti-CD4). *A*, The 1B2$^+$ population of thymocytes was analyzed for CD4 and CD8 expression by three-color staining. *B*, The 1B2$^+$ population of splenocytes was analyzed for CD4 and CD8 expression by three-color staining. Representative results for a single animal of each type are shown. Percentages given in the text reflect the average of six animals of each type.

Rejection of allogeneic tumors is B7 independent

While P815 is a B7-negative tumor, it remained possible that host B7 molecules might be playing a role in costimulating 2C CTL development during a P815 tumor challenge. Such costimulation might be delivered either by host APCs acting in a bystander or *trans* manner or, alternatively, through the processing of an unknown tumor Ag through the CD8$^+$ indirect pathway. To assess whether a role for host B7 molecules exists in the rejection of B7-negative P815, mice were treated with mCTLA4γ3. The efficacy of the mCTLA4γ3 used in the experiments was confirmed by its ability to prevent rejection of the immunogenic P198 tumor in immunocompetent DBA/2 mice (Fig. 6*A*). The ability of B7 blockade to prevent rejection in this model has been taken as evidence for costimulation by host B7 in the rejection of B7-negative tumors (32). Treatment of 2C TCR/RAG mice with an identical course of mCTLA4γ3 or control IgG3 had no effect on the ability of these mice to reject s.c. P815 tumors (Fig. 6, *B* and *C*). RAG$^{-/-}$ mice injected with P815 uniformly grow large s.c. tumors within 3 wk (Fig. 6*B*), while both the mCTLA4γ3 and control treated groups are indistinguishable in their ability to reject these same tumors. Further, the same results were observed independent of the background of the mice (i.e., either RAG-1$^{-/-}$ (Fig. 6*B*) or RAG-2$^{-/-}$ (Fig. 6*C*)). These results illustrate that the observed tumor rejection is independent of both CD4-derived help and B7-mediated costimulation.

Characterization of the effector cell population during tumor rejection

While it was clear that 2C CTLs were necessary for the tumor rejection observed in the TCR/RAG mice, it was not clear how

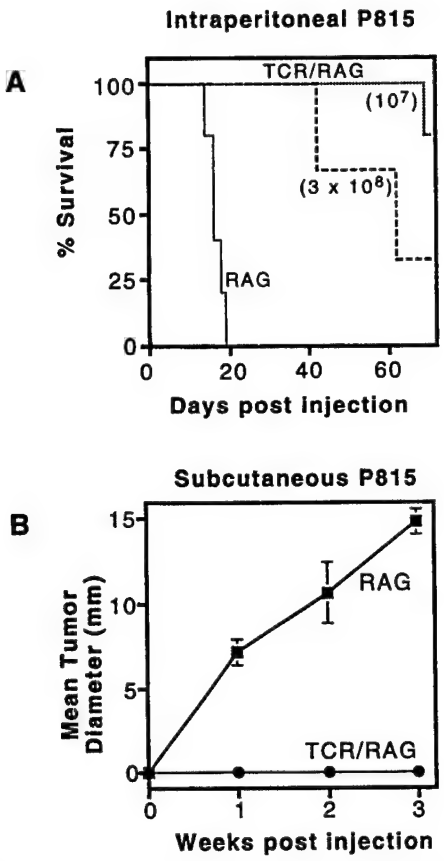

FIGURE 3. Tumor allograft rejection in TCR/RAG mice. *A*, TCR/RAG mice were injected i.p. with either 10^7 P815 cells (n = 5 mice) or 3 × 10^8 P815 cells (n = 3 mice). RAG mice received injections of 10^7 P815 cells (n = 5 mice). Animals were monitored as described in *Materials and Methods* for a period of 10 wk. *B*, TCR/RAG or RAG mice (n = 3) were injected s.c. in the left flank with 10^6 P815 cells in 100 μl and monitored weekly for tumor growth. The mean tumor diameter represents the average of two perpendicular measurements.

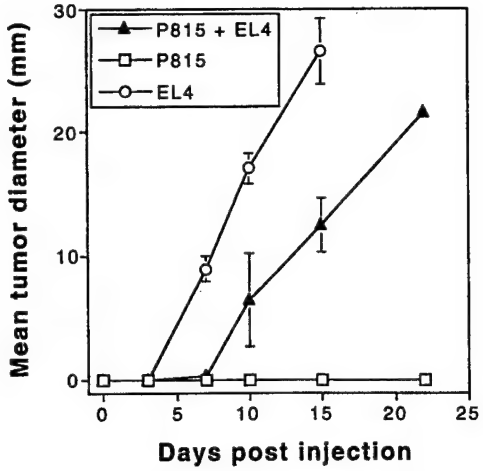

FIGURE 4. Tumoricidal activity in TCR/RAG mice is L^d restricted. TCR/RAG mice were injected s.c. in the left flank with 10^6 EL4 (H-2^b) or P815 (H-2^d) in 100 μl of PBS. Mice receiving coinjection of both cell types received 10^6 of each in a single injection. Mice were monitored as described in *Materials and Methods*. The absence of error bars indicates a SD smaller than the size of the symbol.

these CTLs were accomplishing this task in the absence of any of the established sources of help for a CTL response. To explore the mechanism of tumor rejection, splenocytes and peritoneal exudative cells (PECs) from animals at various times following i.p. P815 injection were examined. Flow cytometric analysis of splenocytes exhibited only very low or background levels of the activation markers CD69, CD25, and CTLA-4 throughout the course of tumor rejection (data not shown). Further, only a low level (12%) of ex vivo cytolysis of P815 target cells was observed by splenocytes on day 4 (Fig. 7). This compares to cytolysis levels of typically >50% for cultured 2C effectors.

To assess whether 2C T cells were activated at the site of the tumor during the period of rejection, PECs were isolated from mice 4 days after i.p. injection of P815 tumor cells. When examined by flow cytometry, PECs showed blastic changes and stained positively for the T cell activation marker CD25, as well as for 2C TCR and CD8 (Fig. 8). In addition to $CD25^+$ blastic 2C T cells (Fig. 8, *B* and *C*), the peritoneal exudate consisted of a subset of smaller $CD25^-$, $CD8^+$ 2C T cells (Fig. 8*A*) as well as a more granular non-T cell population (Fig. 8*D*). The ability of PECs to lyse L^d-bearing target cells (T2-L^d) that were loaded with the peptide QL9 was examined. Ex vivo cytolytic activity of PECs varied from a low of 3% to a high of 40%, averaging 23% in the presence of the peptide QL9 (Table I). A variable amount of peptide-independent lytic activity averaging about one-third of the total lytic activity was observed in the unfractionated PECs. The peptide-dependent lytic activity (about two-thirds of the total activity) was inhibited to the peptide-independent level by addition of the anti-clonotypic Ab 1B2. This result suggests that the remaining peptide-independent lysis is not attributable to killing mediated through the 2C TCR (Table I). The response of TCR/RAG mice to an i.p. injection of BALB/c splenocytes (a B7-positive source of the p2C/L^d Ag) was compared with that to an i.p. injection of P815. The results indicated no significant difference in either CD25 levels or lytic activity of 2C T cells isolated from the peritoneum (data not shown).

In vitro recognition of alloantigen

It has previously been reported that purified $CD8^+$ 2C CTLs do not proliferate when cultured with P815 tumor cells unless exogenous IL-2 is added (41). We sought to confirm this result and determine whether there were any differences that might explain the rejection of P815 tumors by the TCR/RAG mouse. A comparison of the proliferative response to alloantigen in the form of either P815 tumor cells or BALB/c splenocytes (both L^d positive) revealed a marked disparity depending on the nature of the APC (Fig. 9). Proliferation increased as the density of BALB/c APC increased, while proliferation decreased at higher P815 stimulator densities. In the case of both BALB/c and P815, addition of exogenous cytokines increased the proliferative responses. Depending upon the number of stimulator cells used in a single point assay, the ratio of proliferative responses to BALB/c vs P815 ranged from as high as 500:1 to as low as 2:1. When exogenous cytokines were present, this ratio varied from a high of 12:1 to a low of 1:1.5. Thus, it is evident that TCR/RAG splenocytes are indeed capable of proliferation in response to P815 tumor cells (21,000 cpm maximally), but at high densities of stimulators the response to BALB/c far exceeds the response to P815.

In vivo activation by self-MHC-restricted Ag

To determine whether the 2C CTLs were capable of full activation in vivo, soluble antigenic peptides were injected i.p. on 2 consecutive days, and splenocytes were examined for activation on the third day. The peptides included MCMV, a K^b-restricted peptide that is not recognized by the 2C TCR; p2C and dEV-8, peptides that in complex with K^b are recognized with low affinity (K_a ~=

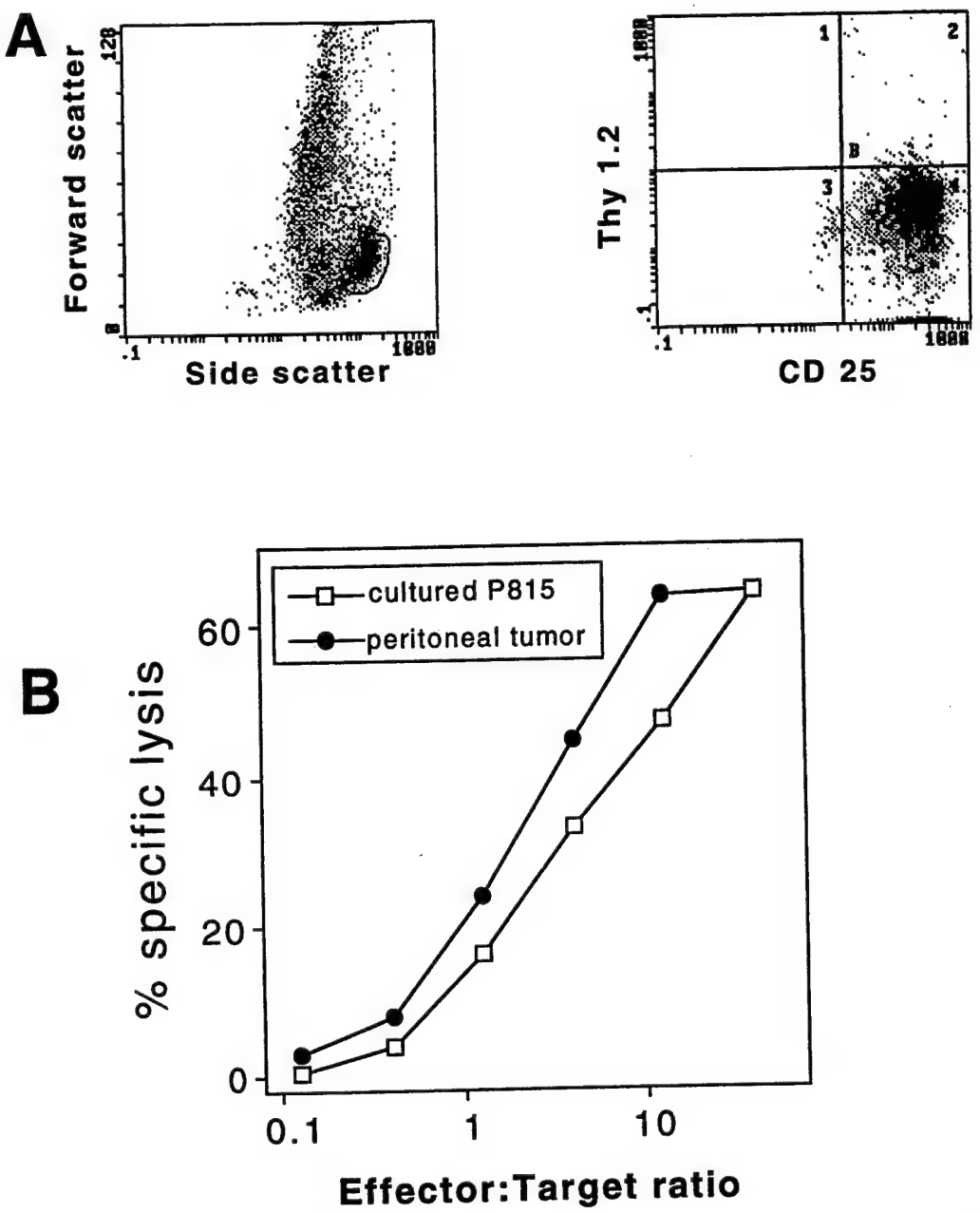

FIGURE 5. Characterization of peritoneal cell population in a TCR/RAG mouse with late tumor reoccurrence. Cells (4.5×10^8) were harvested by peritoneal lavage of an ascites tumor from a moribund mouse injected 69 days earlier with 10^7 P815 cells. *A*, Flow cytometric analysis of the cell populations isolated from the peritoneum of the same animal. The population of macrophages was gated and analyzed for expression of Thy 1.2 and CD25. *B*, Ascites tumor cells that had been in culture for 5 days and cultured P815 were assayed in a standard 4-h ^{51}Cr release killing assay for susceptibility to lysis by in vitro activated 2C CTLs.

3×10^3 and 2×10^5 M^{-1}, respectively) by CTL 2C (21, 22); and SIYRYYGL, a synthetic peptide that was selected from a peptide library based on its ability to sensitize K^b-bearing cells to lysis by 2C (23). Only the SIYRYYGL peptide was able to vigorously activate T cells, as measured by expression of the activation marker CD69 24 h after injection (Fig. 10*A*). Essentially 100% of the $1B2^+$ T cells in the spleen become $CD69^+$ following exposure to this peptide.

To confirm that these activated cells were also capable of cytolysis, the ability of splenocytes from peptide-injected animals to lyse P815 target cells was examined. Again, only the SIYRYYGL peptide elicited splenocytes able to lyse L^d-bearing target cells ex vivo (Fig. 10*B*). Interestingly, injection of the peptide dEV-8, a putative positively selecting peptide for CTL 2C, also failed to produce detectable ex vivo cytolytic activity (Fig. 10*B*) or activation marker up-regulation (data not shown). Thus, 2C CTLs in the TCR/RAG could be fully activated after in vivo Ag challenge, but presumably only the higher affinity peptide/K^b or TCR/peptide/K^b interactions could achieve this activation. It is thought that the 2C TCR recognizes both p2C/L^d and SIYRYYGL/K^b with similar high affinity (23). These results confirm that the 2C CTL precursor cells in the TCR/RAG mice are indeed capable of full and complete activation in response to the appropriate peptide/MHC Ag. It suggests that the low levels of activation observed in the spleen after injection of P815 may be attributable to a lower load of Ag and localization of the Ag to the peritoneum.

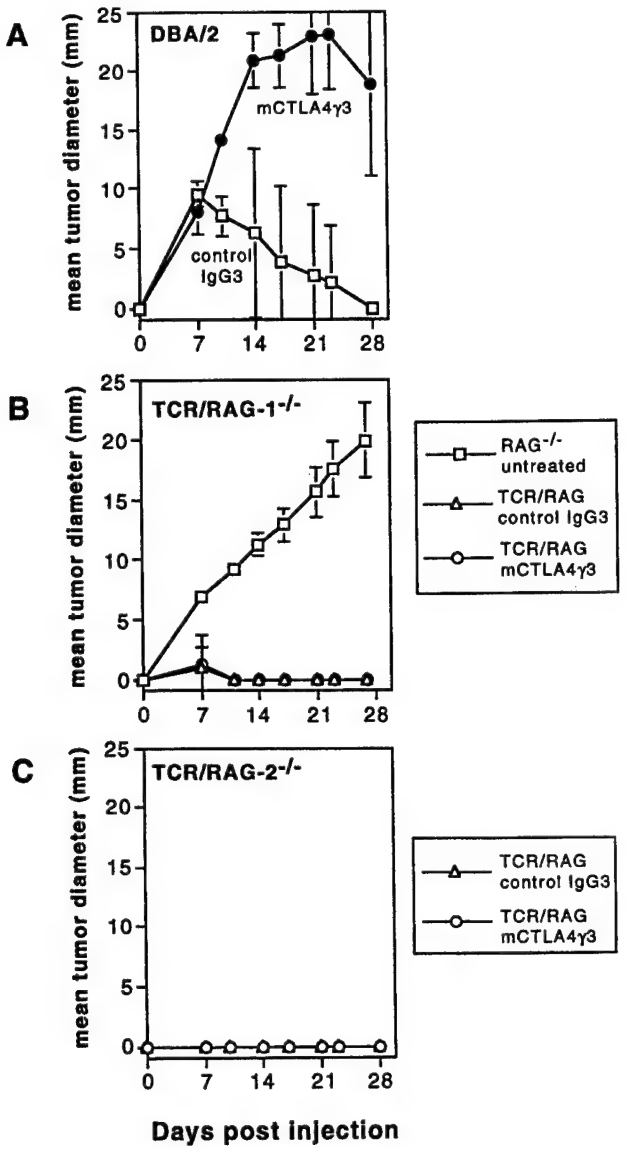

FIGURE 6. Role of B7 molecules in rejection of tumors. Mice were treated with a series of i.p. injections of either mCTLA4γ3 or control IgG3 beginning on day −1 as described in *Materials and Methods*. Tumor cells were injected on day 0 several hours after the second dose of mCTLA4γ3. *A*, DBA/2 mice were injected with 10^6 P198 tumor cells in 100 μl of PBS in the left flank and monitored biweekly for tumor growth. *B* and *C*, 2C TCR/RAG mice on either a RAG-$1^{-/-}$ background (*B*) or a RAG-$2^{-/-}$ background (*C*) were injected with 10^6 P815 tumor cells in 100 μl of PBS in the left flank and monitored biweekly. The mean tumor diameter represents the average of two perpendicular measurements. The absence of error bars indicates an SD smaller than the size of the symbol.

Discussion

The critical importance of costimulation in T cell-mediated responses has been repeatedly demonstrated within the last several years in numerous systems. Nevertheless, several situations have now been described where the most well-characterized costimulation signal, CD28:B7, is not an absolute requirement (16–19). We now present a system where allogeneic tumors are rejected in a B7-independent manner by a mouse that possesses only $CD8^+$ CTLs of a single specificity and that completely lacks B cells or Th cells.

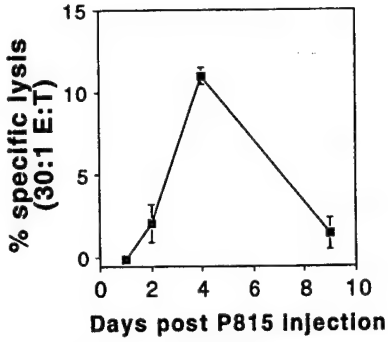

FIGURE 7. Kinetics of T cell activation in TCR/RAG mice during allorejection. Ex vivo cytolysis of splenocytes isolated on various days following tumor injection. Splenocytes were assayed against ^{51}Cr-loaded P815 target cells at various E:T ratios. The lysis at a 30:1 E:T ratio is shown. Each point represents a single mouse. Error bars represent the SD of triplicate wells within the same assay. Fully activated 2C CTLs typically show >50% specific lysis at 30:1 E:T against P815 (data not shown).

Thymic development of 2C T cells appears to proceed along a slightly different kinetic course in TCR/RAG mice compared with TCR mice. Fewer thymocytes mature into $CD4^-CD8^-$ peripheral T cells in the TCR/RAG (5%) than in the TCR (45%). In contrast, there are proportionally twice as many $1B2^+CD4^+CD8^+$ thymocytes in the TCR/RAG thymus as in the TCR thymus. Mice transgenic for a TCR that was specific for MHC-I demonstrated increases in thymic emigrant numbers, but the increase was accompanied by a reduced size of the $CD4^+CD8^+$ thymocyte pool (42). This reduced double-positive pool is not observed when the transgenic 2C TCR is expressed on a RAG background. The explanation for why there are more mature $CD4^-CD8^-$ 2C T cells in the periphery of TCR mice than in TCR/RAG mice is not clear. The $CD8^+$ and $CD8^-$ subsets of 2C cells in the TCR animal are distinct and nonoverlapping and have been purified by others (43). In the TCR mouse, transient expression of endogenous TCR chains during thymic development might reduce the number of transgenic 2C receptors. A reduction in 2C TCR density may lead to altered signaling and a resultant loss of both CD4 and CD8 expression from 2C cells. Variations in the number of TCR per cell required for signaling various T cell functions has recently been described (25, 44).

The fact that 2C TCR/RAG mice are able to reject even large tumor burdens while their corresponding $RAG^{-/-}$ littermates succumb to the tumors indicates that the cell type responsible for this allorejection is the $CD8^+$ CTL bearing the 2C TCR. Our s.c. dose of 10^6 P815 cells is 20-fold greater than the minimal tumorigenic dose used in many syngeneic animal models (31), while our i.p. tumor dose is 10-fold greater still. Because this animal lacks $CD4^+$ T cells or, in fact, T cells that might recognize any other Ag, the CTL response must be independent of CD4 help. What, then, is the source of help necessary for the rejection of these tumors? There are several possibilities. First, it may be that the rejection is made possible by the sheer number of CTL precursors and the high intrinsic affinity ($2 \times 10^6\ M^{-1}$) of the 2C TCR/p2C/L^d interaction, which could abrogate the requirement for costimulation, as has been proposed previously (43). That is, since p2C is immunodominant for L^d, and P815 expresses high levels of L^d, the high epitope density and high TCR affinity may act to overcome the requirement for costimulation. A sufficient number of CTLs may secrete their own cytokines and become activated locally to a level that is capable of initiating the lytic response and controlling the tumor.

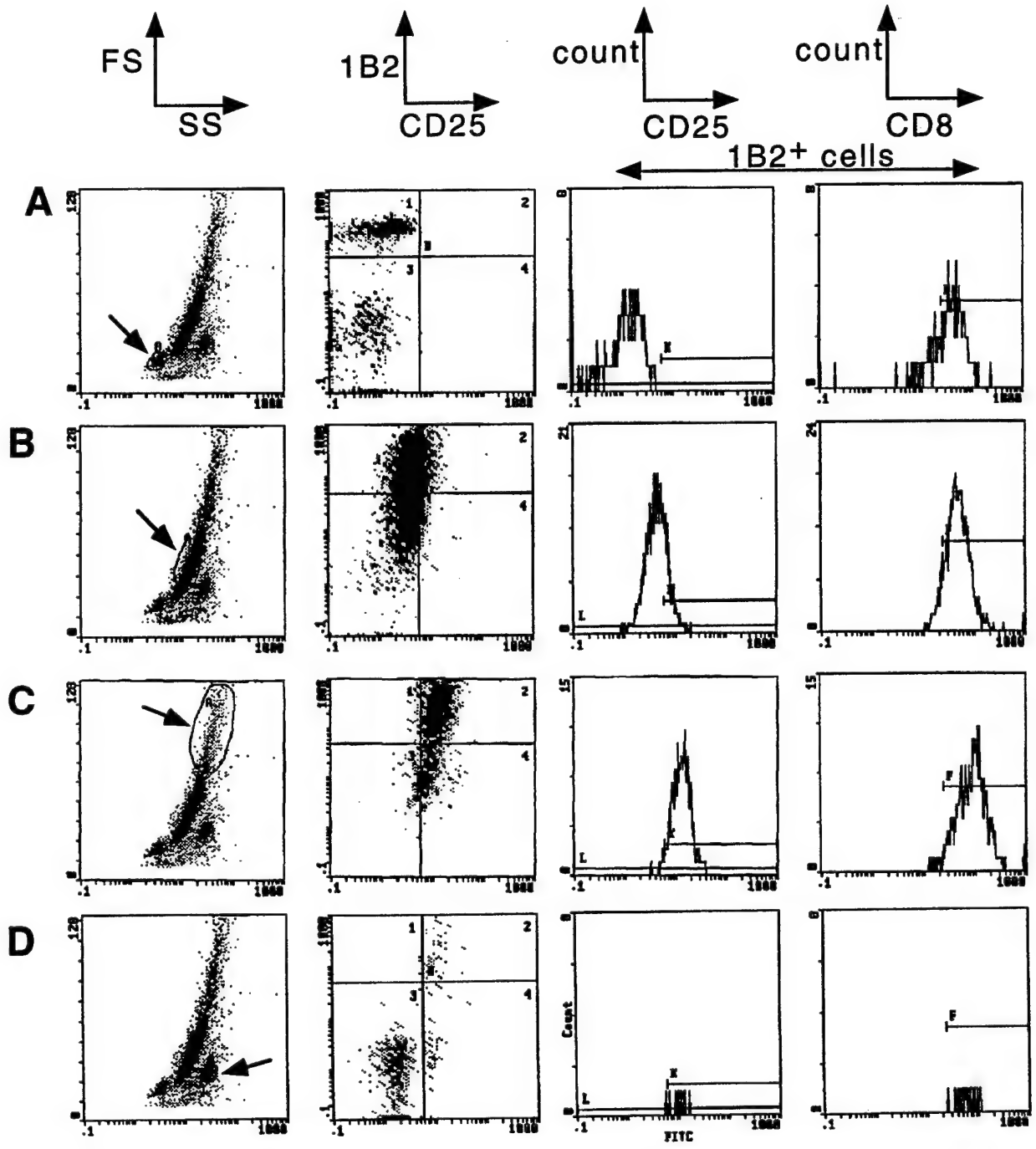

FIGURE 8. PEC populations at the time of tumor rejection. PECs were isolated by peritoneal lavage from mice injected with P815 tumor cells 4 days earlier. Distinct populations were gated upon (gate indicated by arrow) and analyzed for various T cell markers by flow cytometry. *A*, Smaller CD25$^-$ 2C CTL precursors. *B* and *C*, Larger, blastic CD25$^+$ 2C CTLs. *D*, Population of more granular, non-T cells. Results were representative of several mice analyzed.

A second explanation may involve an interplay between T cells and the innate immune system. It has been shown that macrophages and other cell types, such as NK cells, can participate in cytokine cascades that direct a T cell response toward either Th1 or Th2. Moreover, a requirement for NK cells in the induction of alloreactive human CTLs has been demonstrated (45). Such an interplay with the innate immune system operating in the local environment of the peritoneal cavity or an s.c. site might provide the necessary help needed for the development of CTL precursors into lytic CTLs. In Figure 9, the low level of proliferation in response to P815 is shifted nearly fourfold by the addition of exogenous cytokines. Thus, it is possible that other cell types in the local environment might be providing the requisite cytokines. For example, the IL-1β/IL-12/IFN-γ cytokine cascade involves macrophages and NK cells (46). IL-12 secreted by macrophages or dendritic cells is known to be a potent inducer of Th1/Tc1 responses and is probably acting along with Ag to help promote CTL development.

A third possibility is that other costimulatory pathways could be replacing B7:CD28 in producing CTLs capable of tumor cell lysis. P815 is known to express intercellular adhesion molecule-1, which binds the LFA-1 Ag on T cells and can provide costimulatory

Table I. *Lysis of ^{51}Cr-labeled T2-L^d target cells by TCR/RAG PECs*[a]

Mouse	% Lysis by PECs at 30:1 E:T (+QL9)	(No Peptide)	% Peptide Dependent	% 1B2 Inhibition (+QL9)
1	33	20	40	n.d.
2	20	14	30	n.d.
3	40	4.6	89	91
4	33	0.8	97	83
5	2.8	0.4	86	89
6	8.3	3.0	64	54
Average	23	7.1	68	

[a] Effector cells were obtained by peritoneal lavage 4 days following i.p. injection of 10^7 P815 cells. Assays were performed as described in *Materials and Methods*. Maximal 1B2 inhibition was assessed at a 1B2 concentration of 3–10 μg/ml. n.d. indicates that 1B2 inhibition was not examined in the indicated mouse.

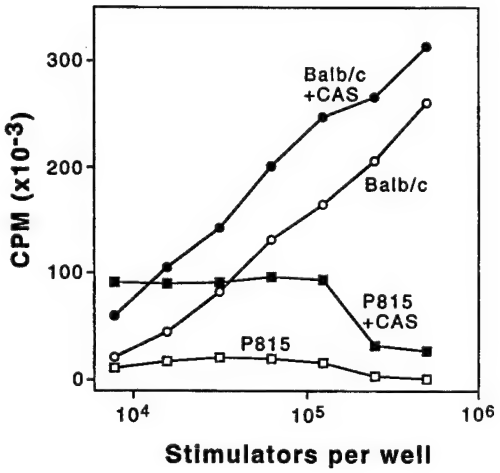

FIGURE 9. In vitro proliferation of 2C CTL precursors in response to alloantigen. Responder TCR/RAG splenocytes were cultured at 5 × 10^4 cells/well with various numbers of allogeneic stimulator cells. Exogenous cytokine-rich supernatant from Con A-treated rat spleens (CAS) was added where indicated at a 10% concentration. Proliferation of stimulators or responders cultured alone yielded background levels <1500 cpm. The maximum proliferation in the presence of P815 without CAS was 21,000 cpm.

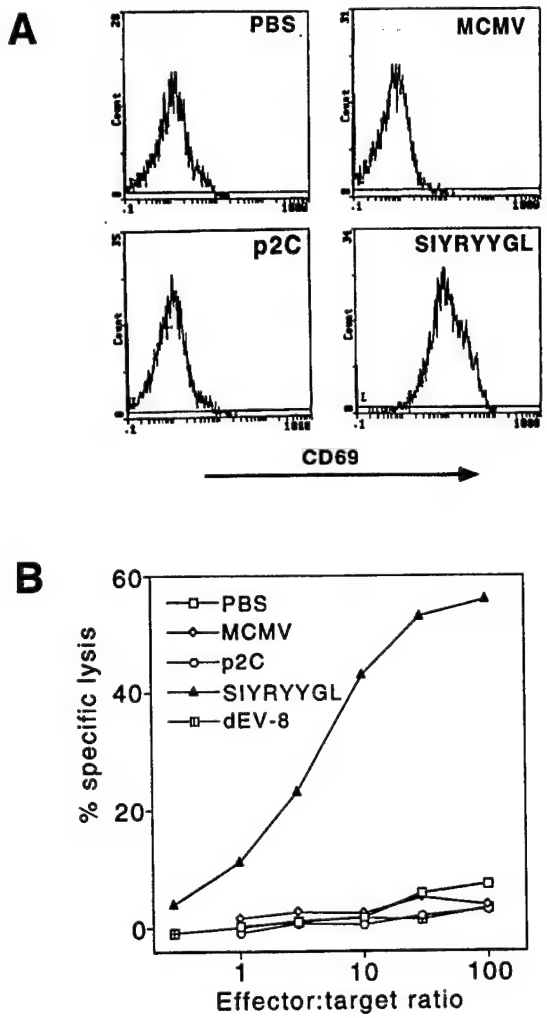

FIGURE 10. Peripheral activation of 2C T cells after injection of self-restricted peptide. *A*, Up-regulation of CD69 T cell activation marker on 1B2$^+$ T cells on day 3 following i.p. injection of antigenic peptide on days 1 and 2. Amounts of peptide injected for the figures shown are 70 nmol for MCMV and p2C and 10 nmol for SIYRYYGL. Injection of larger amounts of SIYRYYGL frequently caused death of the animals secondary to shock-like effects. Peptides were injected i.p. in 200 μl of PBS. *B*, Ex vivo cytolytic activity of splenocytes from mice injected with antigenic peptides in PBS. Spleens were harvested, RBCs were lysed, and splenocytes were assayed at various concentrations against ^{51}Cr-labeled P815 target cells in a standard 4-h assay.

signals to T cells under certain conditions (47, 48). It has been demonstrated that naive T cells from CD28-deficient, OVA-specific Th cell transgenic mice can initiate, but not sustain, an immune response (49). This system differs from our own in that other endogenous nontransgenic T cells are present, and the transgenic T cells are of the CD4$^+$ helper phenotype. Yet another alternate costimulatory pathway that could be substituting for B7:CD28 involves the CD58:CD2 interaction, which has been shown to elicit production of IFN-γ by CD8$^+$ T cells (50).

Each of these possible mechanisms is consistent with the observation that 2C T cells from the site of the tumor at the time of rejection express CD25 and are able to lyse the appropriate tumor cells ex vivo (Fig. 8 and Table I). The fact that the rejection of P815 appears to be independent of B7 does not suggest that B7 molecules are unimportant in the development of a CD8$^+$ CTL response, only that they are not critical for rejection in this system. It may well be that the 2C TCR/RAG mouse could reject much larger tumor burdens if the tumor was transfected with B7. Similarly, B7-positive tumors might be rejected by mice with much lower CTL precursor frequencies.

The fact that several of the TCR/RAG animals eventually succumbed to tumors at a very late stage (Fig. 3*A*) indicates that while tumor control is generally achieved, 2C CTLs did not eliminate the entire tumor load in at least some cases. This result is reminiscent of the persistence of viremia in CD4-deficient or B cell-deficient mice (40, 51, 52), providing support for the idea that CD4$^+$ T cells are important not only for initial CTL priming, but also to help sustain the CTL response (40). A recent report using purified CD8$^+$ 2C T cells has suggested that the maintenance of CTL effector function is dependent on either Th cell-derived cytokines or costimulatory ligands distinct from B7–1 (53). The high number of activated macrophages isolated from the peritoneum (Fig. 5*A*) implies that a source exists for the IFN-γ necessary for the activation of these cells (54, 55). The nature of this source is unknown, although T cells or NK cells could be providing it. The observation that recovered tumor cells and cultured P815 exhibited equivalent susceptibility to killing suggests that the 2C T cells from these animals have become exhausted or peripherally tolerized. In this

regard, the 2C TCR/RAG mice should provide a useful system for investigating CD8$^+$ T cell memory and tolerance.

We did not observe high levels of activation in splenic T cells obtained from mice during the period when rejection was occurring (Fig. 7 and data not shown). It is clear, however, from the in vivo response to SIYRYYGL that the CTL precursors in the TCR/RAG are indeed capable of full activation (Fig. 10). The effector response in the peritoneum of tumor-rejecting mice is characterized by an infiltrate consisting of both 2C CTLs and nonspecific effector cells. It is likely that the effector cell kinetics vary somewhat in both peak strength and timing between various mice. The fact that the lytic activity of the unfractionated PECs is about one-third peptide independent and about two-thirds peptide dependent (Table I) suggests that the 2C T cells may have initiated a Th1-like local response, recruiting and activating macrophages, neutrophils, and NK cells, which may all attack tumor cells. While macrophage-mediated tumor killing is typically measured in an 18- to 24-h assay, at least some of the peptide-independent ^{51}Cr release by unfractionated PECs may be attributable to this cell population. This is consistent with a recent report examining the role of Ag receptor-negative cells in the effector response to an adenocarcinoma (56). Moreover, such a Th1-like response (57) initiated by CD8$^+$ CTLs would explain the delayed onset of EL4 tumors in mice injected with a mixture of EL4 and P815 (Fig. 4). However, the ultimate failure to reject these tumors provides additional evidence for the critical role of the 2C CTLs in the development and maintenance of an antitumor response.

It has recently been demonstrated that CD28 costimulation promotes production of the Th2-like cytokines IL-4 and IL-5 by both CD4$^+$ and CD8$^+$ T cells (58). It is conceivable that presentation of Ag by B7-negative P815 to 2C pCTL might result in less Th2/Tc2 differentiation, allowing a skewing of the T cell response toward the Th1/Tc1 phenotype. Alternatively, another possible explanation for the significant levels of peptide-independent lysis seen by PECs during the period of rejection could rest in the recent observation that Th2-type CD8$^+$ effectors show extensive peptide-independent killing of target cells (59) and also differ from Th1-type CD8$^+$ effectors in their levels of Fas-mediated cytolysis (60). The high numbers of activated macrophages in the animal with tumor recrudescence suggests that a perhaps ineffective Th1-type response may have predominated in this particular mouse. It remains to be determined which of the two pathways of development (Th1- or Th2-like) is followed by naive 2C T cells when challenged with P815 in vivo.

It was previously reported that purified CD8$^+$ 2C CTLs could not proliferate in response to P815 (41). To reconcile our observation that 2C T cells do proliferate weakly in response to P815 with these previous results, it is important to realize that the number of stimulator cells in the assay can have a profound impact on whether proliferation is observed. At the highest densities used in this earlier work (2×10^5/well), we also observed similar low levels of proliferation. However, at lower stimulator cell densities, significant proliferation (up to 21,000 cpm) was detected even in the absence of exogenous cytokines (Fig. 9). This inverse effect of P815 cell density on the proliferative response of purified CD8$^+$ T cells was also described in a previous report (61). Moreover, it has recently been shown that the initial response to large doses of Ag does not require costimulation, but that costimulation plays a key role in prolonging the response (43). These findings are in keeping with the idea that costimulation up-regulates molecules that serve to protect against apoptotic death (11). In fact, the observation that tumors can reoccur in some mice suggests that the ability to mount a prolonged response may have been affected. We are currently examining this possibility.

Allograft rejection by TCR (RAG$^{+/+}$) mice has previously been examined (62), and it was found that female H-Y-specific TCR transgenic mice failed to reject male skin grafts. The authors suggested that this failure to reject might be attributed to a deficiency of CD4$^+$ Th cells secondary to skewing of the repertoire toward CD8$^+$ CTLs. In contrast, we found that the absence of CD4$^+$ cells does not prohibit rejection of allogeneic tumors. These results might be explained by differences in the type of allograft (i.e., skin- vs hemopoietic-derived tumor cells). In this respect, it will be of interest to examine the responses of 2C TCR/RAG mice to skin and cardiac allografts. Alternatively, the explanation may lie in differences in the overall avidity of the TCR/Ag interaction, taking into account both the high epitope density and the intrinsic TCR affinity of the 2C TCR/p2C/L^d interaction and the lower avidity of the H-Y/TCR interaction. Recent work has demonstrated that 2C TCR mice (RAG$^{+/+}$) will vigorously reject cardiac allografts in a situation where all three sources of help for a CTL response are possible (63). The density and load of allograft cells may well be critical in controlling the extent to which CTL precursors either become activated or become peripherally tolerized. It was recently reported that adoptive transfer of 2C T cells (both CD8$^+$ and CD8$^-$) into L^d-bearing SCID mice (where L^d is expressed on all nucleated cells) resulted in a tolerant state achieved through both activation-induced apoptosis and down-regulation of CD8 and the TCR (64). In contrast, when cardiac allografts were transplanted into 2C TCR transgenic mice (RAG$^{+/+}$) treated with rapamycin, T cells showed impaired intracellular signaling but unchanged surface levels of 1B2 and CD8 (63). Clearly, the density of L^d-expressing cells is much greater in an L^d SCID mouse than in the case of cardiac allografts or L^d-bearing tumor cells.

In conclusion, we have described a system in which allorejection occurs in the absence of CD4$^+$ Th cells and independent of costimulation through CD28-B7 interactions. The cell responsible for this allorejection is an MHC class I-restricted, CD8$^+$ CTL of a single specificity acting in the absence of CD4$^+$ cells. Our results suggest that these CD8$^+$ effectors may be mediating their effects at least partly by the induction of a Th1-like local response to the allogeneic tumor cells. These findings highlight the flexibility of the immune response and raise additional questions about an absolute requirement for CD4$^+$ cells in allorejection (7). In addition, the 2C TCR/RAG mice provide a useful system to develop and test agents such as bispecific Abs and other immunomodulating agents that can effectively redirect and/or prolong the activity of a CTL response against target cells.

Acknowledgments

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Targeting T Cells against Brain Tumors with a Bispecific Ligand-Antibody Conjugate[1]

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Abstract

High affinity receptors expressed on the surface of some tumors can be exploited by chemically conjugating the ligand for the receptor and an antibody against immune effector cells, redirecting their cytolytic potential against the tumor. Ovarian carcinomas and some brain tumors express the high affinity folate receptor (FR)[3]. In this report, a transgenic mouse model that generates endogenously arising choroid plexus tumors was used to show that folate/anti-T cell receptor antibody conjugates can direct infiltration of T cells into solid brain tumor masses. An engineered single-chain Fv form of the anti-T cell receptor antibody KJ16 was conjugated with folate, to produce a bispecific agent that was substantially smaller than most previously characterized bispecific antibodies. Folate conjugation to the antibody increased T cell infiltration into the tumors 10-20 fold, and significantly prolonged survival of the mice.

[3]Abbreviations: high affinity folate receptor (FR), major histocompatibility complex (MHC), phosphate buffered saline (PBS), T cell receptor (TCR), single-chain Fv of antibody KJ16 (scFv-KJ16), Staphylococcal enterotoxin B (SEB),

Tumors use a variety of mechanisms to avoid being eliminated by the immune system (1). Some tumors actively suppress immune function by secreting inhibitory cytokines or by killing T cells through the Fas/FasL interaction. Other tumors escape recognition by cells of the immune system. For example, tumors may fail to present peptide antigens in complex with a product of the major histocompatibility complex (MHC), which are essential for recognition by T cells. Tumor cells may also be deficient in costimulatory ligands and adhesion molecules that facilitate recognition and T cell activation. Finally, the early development of tumors may allow the immune system to become tolerant of potential surface antigens on tumors (2).

One potential way to overcome escape due to failures of recognition is to redirect immune cells against tumor cells with bispecific antibodies (3-5). Bispecific antibodies can be constructed to recognize two separate antigens, one on the tumor surface and the other on the surface of an immune effector such as a cytotoxic T cell. Many tumor cells have potential target antigens that are tumor-specific or quantitatively more abundant on tumor cells than normal cells (tumor-associated). Previous work has demonstrated the *in vitro* and *in vivo* effectiveness of bispecific antibodies against a variety of experimental tumors. Several clinical trials have been conducted with first-generation bispecific agents with results that are sufficiently promising to warrant further study of this strategy (6-11).

Despite considerable progress in the design of bispecific antibodies, there remain significant obstacles to their effective clinical use. One of the problems associated with the bispecific antibody approach has been the difficulty in identifying animal models that can mimic human cancers. By analogy with human use, one would like a system that could evaluate how an endogenously arising tumor could be controlled by treatment with bispecific agents that redirect the activity of endogenous T cells. To date, all pre-clinical bispecific antibody studies have used transplanted tumors in either syngeneic rodent systems or xenogeneic systems in which human tumors and lymphocytes are transplanted in immunodeficient mice. This report shows for the first time that a new class of bispecific antibody agents can be used in a transgenic, endogenous tumor model to study T cell-mediated therapies.

The tumor antigen targeted in this study was the high affinity folate receptor (FR), which has been identified on ovarian carcinomas and most choroid plexus tumors and ependymomas (12-14). The high affinity FRs ($K_D \approx 1$ nM) differ from the ubiquitous lower affinity reduced folate carriers ($K_D \approx 100$ μM) that are largely responsible for normal folate uptake (15-17). High affinity FRs were originally identified as tumor-associated antigens using monoclonal antibodies that reacted with ovarian tumor cell lines (18). The presence of FR on ovarian tumors has led to its use as a target for various forms of therapy, including bispecific antibodies (19-21).

The nanomolar affinity of folate for FR suggested that attachment of folate directly to an anti-T cell receptor (TCR) antibody might be a rapid method to generate bispecific antibodies that efficiently target FR-positive tumor cells for lysis by activated T cells. In cytolytic assays, these folate/antibody conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein (22). In order to reduce the size of the bispecific agent, we recently produced folate conjugates of the single-chain Fv of the anti-Vβ8 antibody KJ16 (scFv KJ16), and the folate/scFv conjugates were as effective as the folate/IgG conjugates in cytotoxicity assays against FR-positive cells (23). The 30 kDa folate/scFv conjugate is to our knowledge the smallest bispecific antibody yet reported.

To develop an animal model for testing the folate/bispecific agents, we chose SV11 mice that are transgenic for the SV40 large T antigen gene with the SV40 enhancer (24, 25). SV11 mice develop choroid plexus tumors with 100% penetrance and in a well-defined time period (age of mortality averages 104 days, SD 12). SV40-induced choroid plexus tumors express FR with properties that are very similar to the human FR (26). In addition, flow cytometry and immunohistochemistry of FR on tumor cells indicated that virtually all of the viable cells are FR-positive (data not shown). Given several recent reports that "non-immunological" therapies for treating brain tumors are actually dependent on an intact immune system (thymidine kinase gene therapy (27), anti-sense TGFβ (28), and anti-sense IGF1 (29)), it is reasonable to predict that the folate/bispecific agents may be capable of redirecting the activity of T cells against endogenous brain tumors.

Method

Purification of scFv-KJ16. scFv-KJ16 was solubilized from *E.coli* inclusion bodies and refolded by dilution into 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $CaCl_2$, 1 mM EDTA, 5mM phenylmethylsulfonylchloride, as described previously (30). After concentration by tangential flow (Filtron) the scFv preparation was dialyzed into 20 mM Tris, pH 8.0 and purified by anion exchange chromatography using Q Sepharose Fast Flow (Pharmacia) eluting with 20 mM Tris, pH 8.0, 1 M NaCl. Peak fractions were pooled, concentrated, and dialyzed into phosphate buffered saline (PBS) if used *in vivo* or 0.1 M MOPS pH 7.5 if used for folate conjugation. The purity and activity of the KJ16 scFv preparations were assessed using a competition flow cytometry assay as described previously (30). scFv-KJ16 purified in this manner had an affinity for TCR as reported previously (~120 nM) and was ~95% monomeric as determined by gel filtration (G200).

Preparation of Folate/scFv-KJ16 Conjugates. Folate scFv conjugates were prepared and characterized as described previously (23). Briefly, folate and the crosslinker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, Pierce Chemical Co.) were dissolved in dimethylsulfoxide at a 2.5 molar excess of EDC. After 30 min at room temperature in the dark, a 100-fold molar excess of the activated folate was added to the scFv-KJ16 previously dialyzed into 0.1 M MOPS. After 1 hr at room temperature, the sample was applied directly to a G-25 column pre-equilibrated in 0.1 M MOPS, pH 7.5. Fractions were characterized by absorbance at 280 and 363 nm and peak fractions were pooled, dialyzed into PBS, and filter sterilized. The biological acitivity of each preparation of the folate/scFv-KJ16 conjugate was tested in cytotoxicity assays before use *in vivo*. Folate/scFv-KJ16 conjugate preparations averaged 10 folate molecules per antibody molecule.

Animals. The SV11 strain was obtained from Terry Van Dyke (University of North Carolina). The strain is maintained heterozygous for SV40 large T antigen by mating transgene-positive males

with C57BL/6J females, and determining genotype of offspring by PCR for large T antigen. Transgene-positive males and females have the same survival curves.

***In Vivo* Infiltration of T Cells Assessed by Immunohistochemistry of CD3.** Control mice received two injections of PBS or Staphylococcal enterotoxin B (SEB) (50 μg) followed by PBS. Experimental mice were injected with SEB and scFv-KJ16 without folate (10 μg, i.v., 18 hrs after SEB), or SEB and folate/scFv-KJ16 conjugate (10μg, i.v., 18 hrs after SEB) and sacrificed after 1 or 2 days. Mice receiving SEB and IgG KJ16 or folate/IgG-KJ16 (10 μg) were sacrificed after 2 days. Tissues were fixed by perfusion with acetic acid/zinc/formalin (Newcomer Supply), the brains blocked into 2 mm thick pieces, and paraffin embedded the next day by the University of Illinois College of Veterinary Medicine Histopathology Laboratory. Six blocks per slide were mounted together, allowing estimation of the total number of infiltrating T cells in a tumor. Three micron serial sections were deparaffinized, rehydrated to PBS, blocked with normal goat serum, and incubated overnight at 4° C with primary antibody. The primary antibody was rabbit anti-human CD3 (DAKO), followed by biotinylated goat anti-rabbit secondary antibody (Vector), avidin-biotin-HRP complex (Vector Elite ABC), and nickel-cobalt-enhanced diaminobenzidine (DAB; Pierce) for detection. Slides were counterstained with methyl green, dehydrated and coverslipped. Tumor areas were determined using NIH Image at 25x. Individual T cells were counted at 100x in six planes per brain; the total number of T cells in six planes ranged from 1 (a PBS-treated mouse) to 2,343 (a folate/scFv-KJ16 treated mouse). The number of mice in each condition was as follows: PBS (3), SEB (2), scFv-KJ16 at 24 hrs (2), folate/scFv-KJ16 at 24 hrs (4), scFv-KJ16 at 48 hrs (4), folate/scFv-KJ16 at 48 hrs (4), IgG-KJ16 at 48 hrs (1), folate/IgG-KJ16 at 48 hrs (2).

***In Vivo* Treatment to Test Survival Benefit.** Experimental mice were placed on a low folate diet at age 70 days in order to reduce serum folate levels. Commercial rodent diet contains high folate concentrations, and a reduced folate diet brings serum folate levels down to levels observed in humans (31). We measured serum folate levels in C57BL/6 mice fed normal diet and reduced folate diet, and observed a reduction from 236 ± 12 nM to 56 ± 14 nM within a week, followed by

relatively stable low levels (35 ± 3 nM at 4 weeks). In control experiments with n's = 8 we found that neither the maintenance on low folate diet nor the stress of repeated restraint and injection of PBS affected survival (data not shown). Baseline body weight of mice was obtained at 84 days. With previous cohorts of mice we correlated the loss of body weight due to cachexia and age of death to provide an objective criterion for morbidity. Mice died within 2-3 days of reaching 75% of baseline body weight, so this criterion was used to determine time of sacrifice; mice were also sacrificed if they showed clear neurological symptoms such as vestibular problems (from fourth ventricle tumors) or ataxia and lethargy.

Antibody-treated mice were injected i.p. with 100 μg SEB on day 84, followed 18 hrs later by i.p. injection of either folate/scFv-KJ16 (25 μg in PBS, n = 8) or scFv-KJ16 (25 μg, n = 8). Mice received three additional i.p. injections of antibody (25 μg) over the next 12 days. Control mice (n = 9) were untreated. Mice were weighed and monitored daily for neurological signs.

Statistical Analysis. Infiltration data were analyzed by analysis of variance using SAS JMP software. Survival data were analyzed by Kaplan-Meier estimates using SAS JMP.

Results

To determine whether the folate moiety of the bispecific conjugate could specifically target T cells against SV11 tumors, mice were injected with either scFv-KJ16 or folate/scFv-KJ16 preparations. T cells were activated one day before the injection of the bispecific agent by injecting the superantigen SEB, which activates the Vβ8 population of T cells recognized by KJ16. Mice were sacrificed 24 or 48 hrs after antibody injection and the number of T cells that infiltrated the tumor was evaluated by immunohistochemistry of CD3. In the absence of SEB or bispecific antibody, very few T cells were observed (as few as one or two per brain). SEB alone or SEB in combination with the scFv-KJ16 without folate produced low levels of infiltration (Figure 1B). In contrast, treatment with the folate/scFv-KJ16 conjugate caused a substantial infiltration of T cells into the tumors (Figure 1A). Quantification of T cell density in the tumors confirmed that folate significantly increased T cell infiltration both 24 hrs and 48 hrs following treatment ($p < 0.0001$,

folate/scFv-KJ16 vs. scFv-KJ16, Figure 2). The IgG form of folate/KJ16 produced a moderate level of T cell infiltration at 48 hrs, but less than the single-chain form of the conjugate at the same time point (Figure 2, $p = 0.05$).

Figure 1 and Figure 2 about here.

We then asked whether the folate/single-chain antibody conjugate was capable of redirecting a sufficient number of T cells to have an impact on survival. Our colony of SV11 mice has an essentially identical survival curve to the original description of the strain over 30 generations ago (25), with survival times of 100-105 days for various cohorts. To estimate when to begin treatment, we characterized the histological development of tumors in untreated animals. Consistent with an earlier description (32), a small number of hyperplastic foci usually begin to appear between 70-80 days of age, and then tumors become increasingly anaplastic and grow approximately exponentially over the next 3-4 weeks (Figure 3).

Figure 3 about here.

Based on this characterization of tumor development, mice were treated beginning at day 84 with SEB and either scFv-KJ16 or folate/scFv-KJ16. A single dose of SEB was followed 18 hrs later by an injection of antibody. Three additional injections of antibody were given over the next 12 days. Treatment with SEB and scFv-KJ16 resulted in a significant prolongation of survival compared to untreated controls ($p < 0.05$). However, treatment with SEB and folate/scFV-KJ16 resulted in a further significant enhancement of survival ($p < 0.05$ compared to scFvKJ16, Figure 4). Mean survival times were 100 days for controls, 112 days for scFv-KJ16 treated mice, and 120 days for folate/scFv-KJ16 treated mice.

Figure 4 about here.

Discussion

To our knowledge this is the first report demonstrating that endogenous T cells can be redirected to infiltrate and attack brain tumors. Furthermore, the action of T cells was sufficient to approximately double the survival time following the initial development of tumors in SV11 mice (i.e. ~20 days from day 80 to 100 for untreated mice compared with ~40 days from day 80 to 120 for treated mice). Previous reports have demonstrated tumor infiltrating lymphocytes in established solid tumors after bispecific antibody treatment (33, 34) but few have demonstrated a reduction in solid tumor growth or prolongation of survival (35). The present findings are particularly noteworthy given the difficulties posed by the blood brain barrier and the relative immune privilege of the brain. The transgenic nature of the model makes it additionally challenging to treat; because of the expression of SV40 large T antigen in every choroid plexus cell, each is a potential new focus of tumor development. Thus, even if the existing tumors were completely eradicated, new tumors would presumably arise. Thus it may be necessary to develop a full memory response in T cells in order to cure tumors in this particular model.

Activation of T cells by SEB appears to produce a partial reversal of tolerance, as mice treated with SEB and scFv-KJ16 lived significantly longer than control mice. This finding is consistent with earlier reports that activation of T cells by SEB or an anti-CD3 antibody such as 145-2C11 can generate anti-tumor activity in mice (36, 37). In a separate trial, we treated SV11 mice with 145-2C11 and produced a survival benefit similar to mice treated with SEB and unconjugated scFv-KJ16 in the present experiment (data not shown).

Our results emphasize the importance of both ligand-based targeting of tumors and the use of the smaller scFv form for systemic treatment of solid tumors. The folate conjugate of the scFv form of KJ16 elicited a substantial increase in T cell infiltration and resulted in enhanced survival

that was significantly greater than the scFv without folate. For example, there was ~ 20-fold increase in T cell number at 24 hrs after treatment with folate/scFv-KJ16 and ~10-fold increase in T cell number at 48 hrs after treatment compared to scFv-KJ16 without folate. The single-chain form of the antibody also appeared to be important, as the folate/scFv-KJ16 produced an approximately three-fold higher number of T cells compared to the IgG form of the folate/KJ16 conjugate at 48 hrs. Thus either IgG or scFv forms of the folate conjugates can retarget T cells against the tumor, but the single-chain antibody may provide better access of the antibody to T cells or better tumor penetration from systemic circulation (38). These measurements provide a useful guide to the possible relationship between the extent of T cell infiltration into a tumor and the benefit regarding survival. Extensions of these studies should allow us to evaluate if sustained or more extensive T cell infiltration can lead to greater survival rates.

Another important aspect of this study regards the etiology of SV11 tumors (i.e. SV40 large T antigen interferes with p53 and pRB (39)) and their similarity with some human brain tumors. SV40 has been isolated from pediatric choroid plexus tumors and ependymomas (40, 41), although the significance of finding SV40 in these tumors and other types of human tumors is still debated (42). SV40-induced tumors are immunogenic if transplanted into congeneic C57BL/6 mice (43, 44), but SV11 mice are apparently immunologically tolerized to the tumors during development, as are other SV40 transgenic strains (2). We have transplanted the choroid plexus tumors to a subcutaneous location in SV11 mice, and the tumors are not rejected (data not shown). At this point we have not determined whether either of the treatments that prolonged survival generated a tumor-specific T cell response. It may be that the folate-conjugation and targeting of T cells involves both redirected killing of tumor cells and the generation of tumor-specific T cells that have overcome tolerance, enhancing the process that occurred in animals treated with SEB and scFv-KJ16.

We have investigated the relationship of the blood brain barrier (or blood cerebrospinal fluid barrier) to this treatment strategy. As the tumors develop, there are multiple foci in the lateral ventricles and fourth ventricle. The smaller tumors have an intact barrier (as revealed by the

absence of murine IgG and exclusion of Evans Blue/albumin) whereas the larger tumors have lost the barrier. Although there is heterogeneity in the density of the T cell infiltrate in different foci, it is only partly accounted for by the blood brain barrier. For example, there are definite small tumors with an apparently intact blood brain barrier that also have infiltrating T cells. Somewhat surprisingly, we observed few T cells associated with regions of normal choroid plexus, which also expresses the high affinity folate receptor (13, 26). Consistent with this finding, there have been no signs of obvious neurological problems in tumor-bearing or normal C57BL/6 mice treated with the bispecific agents described in this study, nor apparently, in ovarian cancer clinical trials using conventional anti-FR bispecific antibodies (19-21). It may be that the blood CSF barrier is intact in the choroid plexus, and the tumor vasculature is partially compromised even in small tumors. In this regard, there is a substantial body of literature supporting the notion that activated T cells can penetrate the blood brain barrier (45, 46) but penetration is enhanced through a partially disrupted barrier (46). This ability to penetrate the blood brain barrier suggests that strategies to enhance T cell activity in brain tumors may have advantages over strategies that rely on the penetration of antibodies alone.

The general approach of coupling a small high-affinity ligand to antibodies against immune effector cells should be widely applicable to any type of cancer where a high-affinity ligand against a tumor-associated molecule can be identified. The folate/antibody approach may work more effectively against tumors such as ovarian carcinomas that do not have the complication of the blood brain barrier and that may be more accessible by T cells. It is also likely that the ligand-targeting approach described here can be used to enhance other aspects of a T cell response. In particular, folate conjugation to antibodies against T cell co-stimulatory receptors such as CD28 may elicit enhanced T cell activation at the site of the tumor, thereby combining activation and targeting while limiting systemic toxicity (47, 48).

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Figure Legends

Figure 1. Infiltration of choroid plexus tumor by T cells following treatment with folate/anti-TCR bispecific antibody.

A. Folate/scFv-KJ16. Immunohistochemistry of T cell marker CD3 following treatment of SV11 mice with SEB (50 μg i.p.) and folate/scFv-KJ16 antibody (10 μg i.v.). Extensive infiltration of darkly staining T cells is observed. Tumor cells are counterstained with methyl green.

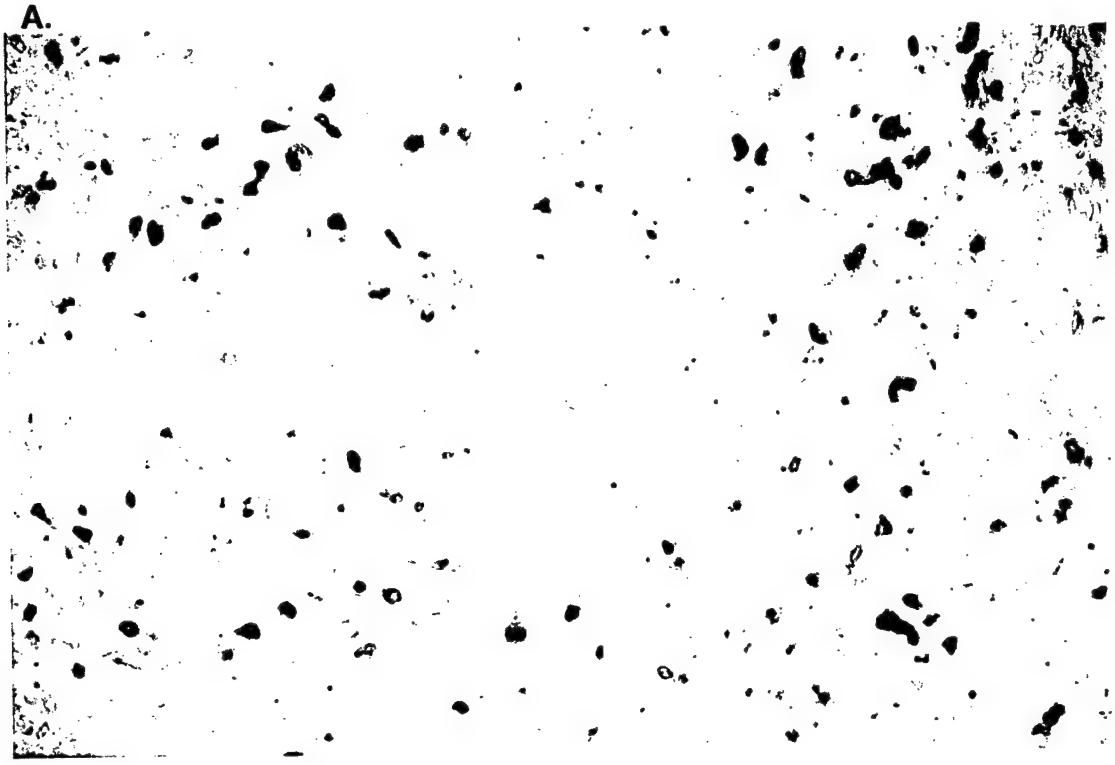
B. scFv-KJ16. Immunohistochemistry of CD3 following treatment with SEB (50 μg i.p.) and scFv-KJ16 antibody (10 μg i.v.) reveals minimal T cell infiltration when folate is not conjugated to the antibody.

Figure 2. Quantitation of T cell density in tumors following treatment with various anti-TCR antibody preparations. Controls were injected with PBS vehicle or superantigen SEB. Experimental mice were injected with SEB followed by one of two forms of the anti-Vβ8 TCR antibody KJ16, either single-chain Fv (scFv) or IgG. Each of the two forms of KJ16 was either conjugated with folate or non-conjugated. Mice receiving the scFv antibodies were sacrificed 24 hrs or 48 hrs following antibody treatment, and mice receiving IgG antibodies were sacrificed 48 hrs following antibody treatment. Conjugation of the antibody with folate increased the infiltration of T cells into the tumor in all cases, with the highest density of T cells observed 48 hrs following the scFv form of the folate conjugate.

Figure 3. Development of tumors in SV11 mice. Mice were sacrificed at various ages, either while still healthy (non-moribund, circles) or moribund by neurological or body weight criteria (triangles). Tumor volume was calculated from tumor area, determined with NIH Image from evenly spaced sections throughout the brain. Tumors develop rapidly after approximately day 80.

Figure 4. Prolongation of survival of SV11 mice following antibody treatments. Experimental mice were treated with SEB (100 μg i.p.) at day 84 followed over the next 13 days by four treatments with either scFv-KJ16 (25 μg i.p.) or folate/scFv-KJ16 (25 μg i.p.). Mice treated with SEB/scFv-KJ16 lived longer than controls ($p < 0.05$) and mice treated with SEB and the folate conjugate of scFv-KJ16 lived longer than mice treated with the non-conjugate form of the antibody ($p < 0.05$).

A.



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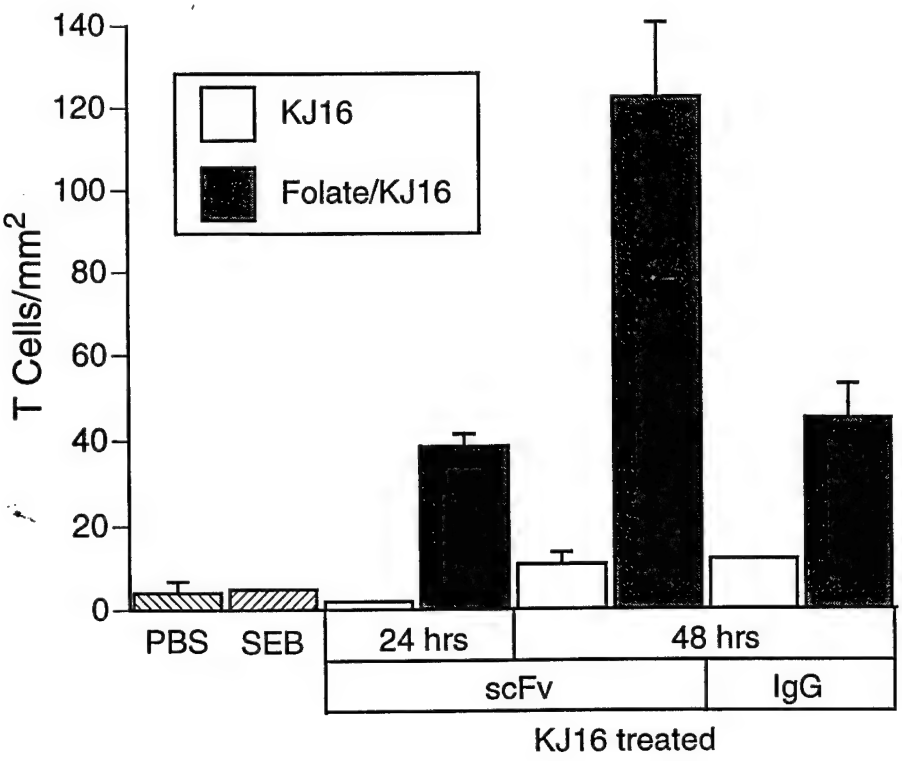
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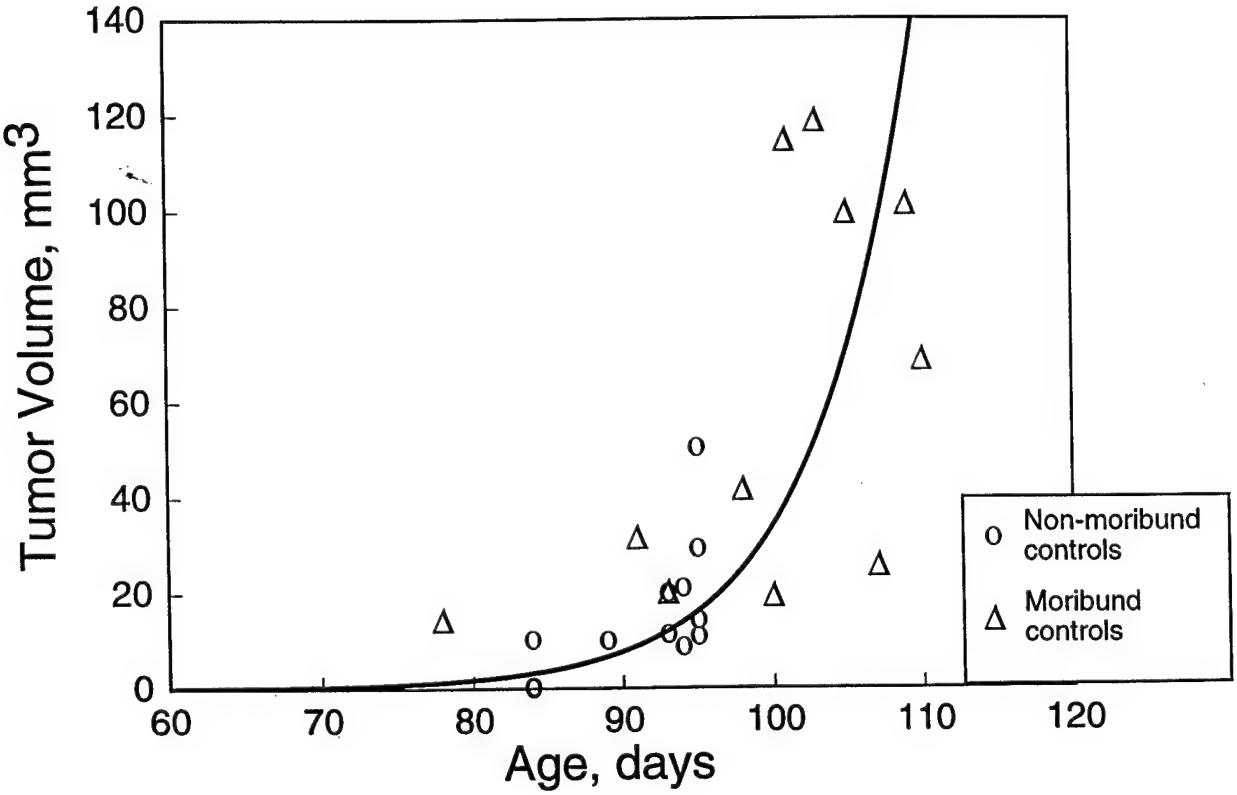
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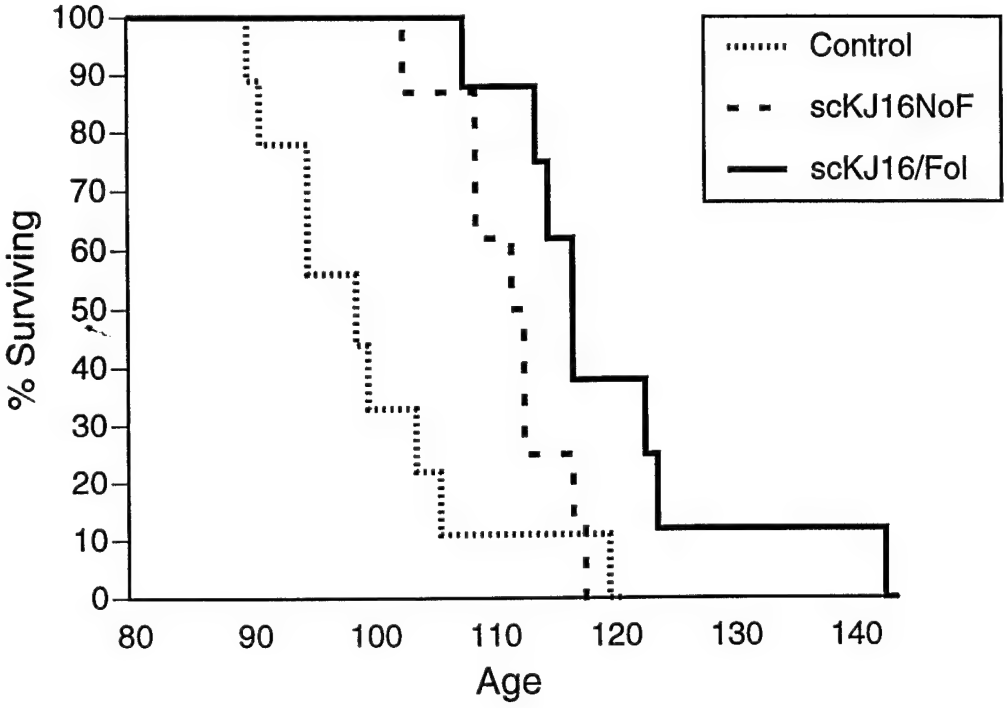
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A Transgenic Model to Test Bispecific Antibody Targeting of Endogenous T Cells Against Human Tumors

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Running Title: Bispecific Antibody Therapy in a Xenotransplant Model

Key Words: bispecific antibodies, folate receptor, cytotoxic T lymphocytes, single-chain antibody, transgenic mice.

Abstract

Recently a variety of immunological approaches to cancer treatment have been explored. These include strategies designed to enhance or redirect the activity of T cells against tumors. Bispecific antibodies comprise a class of agents that are capable of redirecting T cells by binding to a tumor antigen and the T cell receptor. *In vivo* testing of bispecific antibodies against human tumors has been limited to the use of immunodeficient mice that receive the bispecific agent, activated human effector T cells, and human tumor cells. In this report we characterize TCR transgenic/RAG-1 knockout mice (TCR/RAG) as a model that will allow endogenous T cells to be redirected against transplanted human tumors. The findings show that TCR/RAG mice 1) accepted transplants of human tumors, including the folate-receptor-positive tumor line KB; 2) contained endogenous CTLs that could be activated *in vivo* with a peptide recognized by the transgenic TCR; and 3) rejected KB tumors after treatment of mice with the T cell-activating peptide and bispecific agents that contained folate covalently linked to an anti-TCR antibody. Successful rejection was achieved with either folate/Fab fragment conjugates or folate/scFv conjugates. The growth of established tumors was delayed after treatment of mice with these agents, but the efficacy was less than when tumors were treated at the same time as transplantation. TCR/RAG mice should provide a well-defined, monoclonal lymphocyte system for the discovery of T cell modulating agents that improve tumor targeting potential.

Introduction

Over the past decade, bispecific antibodies have been used in attempts to redirect the immune system against a variety of different types of cancer. Bispecific antibodies that are designed to redirect the activity of T cells typically recognize both a tumor antigen and the T cell receptor (TCR/CD3) complex (1, 2). *In vitro* and *in vivo* studies with many different tumor cell types have demonstrated the potential of this approach. However, results have also shown that optimal activity of these agents will most likely require combined use with T cell activating agents such as anti-CD3 and anti-CD28 antibodies or superantigens (e.g. Staphylococcal enterotoxin B, SEB) (3-10). These findings are not surprising in light of the current understanding of the signals that are required to induce resting T cells to fully activated T cells. That is, the co-stimulatory molecule CD28 (11, 12), and perhaps LFA-1 under some circumstances (13, 14), must be triggered in order to activate T cells and these signals are not normally delivered in the presence of anti-tumor/anti-TCR agents alone.

Even in combination with T cell activating agents, bispecific antibody therapy of solid tumors has been particularly difficult (15-17). Part of the problem has been the inability to induce sufficient T cell infiltration into a solid tumor or to sustain the activity of T cells at tumor sites. Adequate pre-clinical study of anti-human bispecific antibodies in these respects has been impossible because these agents must be tested in immunodeficient animal models, due to the barriers of xenotransplantation. Accordingly, the bispecific antibodies are typically administered together with activated human T cells and the human tumor into either nude or SCID mice. In these animal models, one cannot evaluate the complexities that are associated with the *in vivo* activation of T cells, the migration of endogenously activated T cells, or the ability to induce and sustain such T cells at tumor sites.

The recent development of transgenic and knockout mice has provided an opportunity to design novel animal models that could potentially be used in these studies. For example, genetically-defined immunodeficient mice were produced by knocking out the recombination-activating-genes 1 (18) and 2 (19). These mice are unable to rearrange immunoglobulin or T cell

receptor genes and therefore mature lymphocytes of the B and T cell lineages do not develop. In contrast to these immunodeficient mice, TCR transgenic mice express the rearranged α and β chain genes from a specific T cell clone and therefore contain predominantly a monoclonal population of T cells. For example, mice that are transgenic for the TCR from the mouse CTL clone 2C bear the same TCR on ~ 60 to 90% of their $CD8^+$ T cells (20, 21). However, the remaining T cells express endogenous TCR and thus these mice are capable of mounting an immune response against various antigens. Because of this, 2C TCR transgenic mice will not accept allogeneic or xenogeneic tumor transplants.

As the 2C CTL does not itself recognize human tumors, we reasoned that 2C TCR transgenic mice crossed with RAG-1 knockout mice could provide a useful system to evaluate the parameters involved in optimal targeting of endogenous T cells against transplanted human tumors. This report characterizes the 2C TCR/RAG-$1^{-/-}$ system as a model for the treatment of human tumors with bispecific agents that recruit endogenous T cells against the tumor.

This system also has the advantage that CTL clone 2C has been studied by many investigators to identify the ligands that the 2C TCR recognizes and to explore the requirements for activation. Several peptides that are recognized by the 2C TCR when bound to the negatively-selecting class I MHC (L^d) or the positively-selecting class I MHC (K^b) have been described. Initially, the octomer peptide p2C was identified as the natural peptide recognized by 2C when bound to L^d (22). QL9, a nonomeric variant of p2C, was shown to be even more effective at stimulating CTL 2C when bound to L^d (23). More recently, two peptides dEV8 (24) and SIYRYYGL (25), when bound to K^b, were shown to be recognized by CTL 2C. There is now also considerable information about the ability of the peptides p2C and QL9 and anti-CD3 and anti-CD28 antibodies to stimulate resting T cells isolated from 2C TCR transgenic mice (e.g. (26-29)).

The human tumor model chosen to explore the TCR/$RAG^{-/-}$ system is the folate receptor-positive line KB. The folate receptor (FR) is expressed on over 90% of ovarian tumors and conventional bispecific antibodies against this tumor antigen were among the first to be tested clinically (30, 31). The KB tumor has also been used *in vitro* to evaluate a variety of novel

therapeutics that are directed against the high affinity FR (32-40). These potential therapeutics contain folate attached to cytotoxic moieties or to imaging agents. Folate attached to anti-TCR antibodies can redirect the activity of T cells against FR+ tumors (41). Recently, we have shown that the smallest bispecific antibodies yet described could also be engineered by attaching folate to the Fab fragment and scFv forms of an anti-TCR antibody (KJ16) (42). As an extension of these studies, we show in this report that these folate conjugates are also potent agents in the CTL-mediated lysis of the human KB tumor. Finally, the folate conjugates are shown to be effective *in vivo* as surrogate T cell antigens on transplanted KB tumor cells. The findings show that the TCR/RAG$^{-/-}$ model can now be used to guide the discovery of improved T cell-specific agents. As such, this system should facilitate more rapid and effective pre-clinical development of various T cell modulating drugs.

Materials and Methods

Antibodies. KJ16 is a rat IgG antibody that is specific for the Vβ8 region of the TCR (43). The Fab fragments and KJ16 scFv were generated and purified as described previously (44). Briefly, scFv was refolded from inclusion bodies and purified by Q-sepharose ion exchange chromatography. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously (40, 41). Each conjugate preparation was analyzed for biological activity in a cytoxicity assay prior to use in mice.

SIYRYYGL Peptide. The SIYRYYGL peptide (25) was synthesized on an Applied Biosystems 430A instrument (Foster City, CA) using standard F-moc chemistry at the University of Illinois Biotechnology Center (Urbana, IL). The peptide was purified by reverse phase HPLC using a C18 column and analyzed for purity by mass spectrometry and for concentration by quantitative amino acid analysis.

Mice. Mice that express the αβ TCR transgenes (20, 21) from the 2C CTL clone were crossed with the recombination activation gene-1 knockout mice (RAG-1$^{-/-}$) (The Jackson Laboratory, Bar

Harbor, ME) (18) to produce the TCR/RAG line of mice. Both the 2C TCR transgenic mice and the RAG-$1^{-/-}$ mice were on an H-2^b background. Mice were maintained in barrier cages at the University of Illinois animal care facility. Experiments were performed on mice between 6 and 12 weeks of age.

Cell lines. KB was derived from a human squamous cell carcinoma (45) and expresses high levels of folate receptor (32, 37). These cells were maintained in folate-free DMEM, containing 10% fetal bovine serum. P815, a DBA2-derived H-2^d mastocytoma tumor line (46) against which the T cell clone 2C was initially selected, was maintained in complete RPMI 1640 that contained 10% fetal bovine serum (47). RMAS, an H-2^b tumor line that does not express the high affinity folate receptor, was maintained in complete RPMI 1640 that contained 10% fetal bovine serum. CTL clone 2C was maintained in complete RPMI 1640 that contained 10% fetal bovine serum, 10% culture supernatant from rat splenocytes stimulated with concanavillin A, and mitomycin C treated BALB/c splenocytes as stimulator cells.

Cytotoxicity assays. Cytoxicity was examined in standard ^{51}Cr release assays. Briefly, target cells were labeled with ^{51}Cr for one hour at 37°C. 10^4 target cells per well of a 96-well plate were incubated with various numbers of effector cells for four hours. Supernatants were harvested and assayed for specific lysis according to the formula: % specific lysis = (cpm experimental - cpm spontaneous)/(cpm maximal - cpm spontaneous). Spontaneous lysis was less than 20% of maximal lysis.

Analysis of redirected lysis used a slightly modified protocol. Folate free DMEM media was used with KB cells as the target. Cultured 2C or *in vitro* activated T cells were used as effector cells. The folate/antibody conjugates were added to triplicate wells at various concentrations diluted in folate-free medium. In some cases, free folate was added to a final concentration of 1.2 μM.

In vivo treatments.

SIYRYYGL peptide activation. Mice were injected i.p. with SIYRYYGL peptide (25) in 200 µl of phosphate buffered saline (PBS). For experiments with the KB tumor, the peptide was given 24 hours prior to each antibody administration. All peptide and antibody agents were sterile filtered prior to *in vivo* use.

KB transplantation. For subcutaneous tumor transplants, each mouse was anesthetized and hair removed from the injecton site before KB cells (10^7, 3×10^6 or 2.6×10^6) in 200 µl PBS were injected. These injections were made on the left side of the back near the scapula. Following the injections, Vernier calipers were used to take 2 perpendicular tumor measurements twice weekly. Measurements were averaged to determine mean tumor diameter. As the tumors reached a mean diameter of 20 mm, animals were euthanized with CO_2. For intraperitoneal tumor transplants, 5×10^6 cells in 1 ml of PBS were injected. These mice were monitored daily and euthanized as the tumors enlarged the abdomen and mice became moribund.

Bispecific conjugate therapy. All mice, except the no SIYRYYGL group, were treated with 2.5 nmol SIYRYYGL peptide on day -1 and tumor cells on day 0. KB cells were suspended in PBS alone or in PBS containing the KJ16 antibody preparation. Antibody treatment groups included: folate/Fab (10 or 20 µg), folate/scFv (1, 10 or 20 µg). Control antibody groups included scFv without attached folate (20 µg) and Fab fragments without attached folate (10 µg). For treatments initiated on day 0, KB cells were mixed with the antibody prior to injection. A second SIYRYYGL peptide treatment was administered on day 3, followed on day 4 with the appropriate antibody (i.p. 200 µl for s.c. transplants and 1 ml for i.p. transplants). Additionally, the i.p. established treatment groups were injected with KB cells 2 days prior to antibody therapy. These "established" tumor groups received antibody treatments on days 2 and 6, preceded by peptide activation on days 1 and 5.

Immunohistochemistry. Tumor samples were fixed by immersion in acetic acid/zinc/formalin (Newcomer Supply, Middleton, WI), paraffin embedded, and sectioned at 3 microns.

Immunohistochemistry was performed on pepsin treated tissue sections using a rabbit polyclonal antibody against the mouse FR, that cross-reacts with the human FR (kindly provided by Dr. Kevin Brigle, Virginia Commonwealth University), at a 1:500 dilution. Secondary antibody was a biotinylated goat anti-rabbit antibody, followed by avidin-biotin-HRP (Vector Elite; Vector, Burlingame, CA), metal-enhanced DAB (Pierce, Rockford, IL) and methyl green counterstain. Controls included omission of primary antibody and a comparison of the immunoreactivity of the FR-negative mouse cell line RMAS.

Statistics. Differences in survival between treatment groups was determined using the Kaplan-Meier survival method within the SAS JMP software.

Results

Growth of human tumors in TCR/RAG mice. RAG-1 knockout mice have been shown to lack B and T cells, although they do not exhibit the leakiness of SCID mice ((18) and unpublished observations). We have observed that RAG-$1^{-/-}$ mice are capable of accepting various human tumor xenografts (Figure 1 and data not shown). In order to develop an animal model that would allow endogenous T cells to be retargeted against such transplanted human tumors, a 2C TCR transgenic mouse was crossed with a RAG-$1^{-/-}$ mouse.

TCR/RAG$^{-/-}$ progeny from backcrossed mice exhibit an immune system in which essentially 100% of the peripheral T cells bear the 2C TCR and 95% are $CD8^+$ (Manning et al., manuscript submitted). Since CTL clone 2C is incapable of recognizing any human tumor line that has been tested ((48) and unpublished results), we reasoned that 2C TCR/RAG transgenic mice might also accept some human tumor xenografts, as do RAG-$1^{-/-}$ mice. Of several different human tumor lines tested (SKBR-3, SKOV-3, BT474, KB) all except SKBR-3 grew consistently as subcutaneous transplants (Figure 1 and data not shown).

The folate receptor positive line KB was selected for further study, in order to evaluate if various bispecific folate/antibody conjugates (41, 42) would be capable of redirecting the activity of the endogenous 2C CTL against the human tumor. Immunohistology of KB tumors that grew

after intraperitoneal or subcutaneous injection confirmed that these tumors continue to express the folate receptor after transplantation in TCR/RAG mice (Figure 2). Folate receptors also appeared to be distributed uniformly at the surface of KB cells. As expected, a transplanted FR-negative tumor RMAS was negative with the anti-FR antibody reagent (Figure 2).

KB tumor xenografts are locally aggressive, but appear to be only weakly metastatic. The tumors rapidly outgrow their blood supply, resulting in relatively well demarcated margins with a necrotic core. Subcutaneous tumor grafts rarely grew outside of the subcutaneous space. Intraperitoneal growth (see below) usually involved several independent foci adherent to the peritoneal wall or organs. These tumors did not appear to be metastatic beyond the abdomen but occasionally invaded the liver or diaphram.

In vivo activation of T cells in TCR/RAG mice. Various studies have shown that initial treatment of mice with a T cell activating agent enhances the tumoricidal activity of bispecific antibodies (4, 6, 7, 9, 49, 50). Two agents that have been used for this purpose are the anti-CD3 antibody 2C11 or the superantigen SEB. In 2C TCR/RAG mice it is also possible, in principle, to use the peptide SIYRYYGL as an activating agent because it is recognized by CTL 2C in the context of the K^b class I product (25) that is present in these TCR/RAG mice. To demonstrate the effect of *in vivo* treatment with SIYRYYGL peptide, splenocytes from mice that received i.p. injections of the peptide were assayed directly *ex vivo*, without further culturing, using the target cell P815 (47). CTL-mediated activity peaked at 1 day after injection and significant activity could be detected up to 3 days after peptide treatments (Figure 3A). We have recently shown that the injection of other K^b-binding peptides that are not recognized by CTL 2C do not induce T cell activation in the TCR/RAG mice (Manning et al., manuscript submitted).

In order to assess the feasibility of repeated activation, we compared the *ex-vivo* lytic activity from animals that received a single injection of peptide with animals that received two injections. The two injections were spaced either 1 day (Figure 3A) or 4 days (Figure 3B) apart. No significant decline in T cell cytolytic activity was observed in animals that had received prior

injections of the peptide, indicating that mice could be treated with multiple doses of bispecific antibodies following T cell activation with peptide.

To confirm that peptide-activated T cells are not alone sufficient for rejection of KB tumor, mice received SIYRYYGL peptide one day prior to KB transplantation. KB tumors grew equally well in peptide-treated and untreated mice (Figure 4). This finding further supports previous observations that the 2C TCR does not cross-react with human tumor antigens.

T cell lysis of KB cells mediated by folate/anti-TCR antibody conjugates. We have shown previously that folate conjugates of various anti-TCR antibodies can redirect CTLs to lyse FR-positive mouse tumor cells (41, 42). In order to determine if the FR-positive human tumor KB could likewise be recognized and killed by mouse T cells in the presence of these conjugates, KB was used as a target cell in cytotoxicity assays. Effector cells included either the CTL clone 2C (Figure 5A) or peptide-activated TCR/RAG splenocytes (Figure 5B). The folate conjugates examined in these experiments included folate/Fab fragments and folate/scFv fragments of the anti-Vβ8 antibody KJ16 (42). Both conjugates were capable of mediating the lysis of KB cells by either CTL 2C (Figure 5A) or activated splenocytes (Figure 5B). The differences in the maximal lysis observed in these experiments appears to be a function of the potency of the effector cells and not of either the state of the KB cells or the folate conjugates (e.g. different cultures of activated splenocytes can yield different maximal killing with the same conjugate and targets).

The lysis induced in the presence of the folate conjugates was mediated through the folate receptor, as evidenced by the complete inhibition that was observed in the presence of excess folate (Figure 5A). In addition, unlabeled Fab or scFv fragments did not mediate lysis (data not shown). Together, these results prompted us to evaluate *in vivo* tumoricidal activity of the folate conjugates.

Bispecific conjugate-dependent rejection of KB tumors by TCR/RAG mice. The folate conjugates were tested *in vivo* against KB tumor cells that were transplanted subcutaneously or intraperitoneally. The i.p. model was evaluated in addition to the s.c. model, as growth of the KB tumor i.p. provides a more realistic model for adjunctive therapy of disseminated

ovarian carcinomas. As indicated above, over 90% of ovarian cancers express the folate receptor (30, 31) at levels that are similar to the KB tumor cell line (32).

In a preliminary experiment, mice were injected s.c. in the flank with 3 x 10^6 KB cells that were mixed with PBS (n = 3) or with 20 μg of the folate/Fab conjugate (n = 3) prior to injection. All mice received SIYRYYGL peptide one day prior to transplantation in order to activate T cells. As shown in Figure 6A, the KB tumor grew uniformly in all three of the control mice, but was completely rejected in two of the mice where tumors were mixed with the folate/Fab conjugate. The other mouse exhibited a 6 week delay in the growth of the tumor. Once established, this tumor grew at a rate that was similar to tumors from the control mice. Statistical analyses of these and subsequent s.c. experimental groups are shown in Table 1.

Additional experiments were performed to confirm the specificity of the conjugate-mediated rejection and to determine if it could be extended to the use of folate/scFv conjugates. As shown in Figure 6B, KB tumors grew equally well in mice when they were pre-treated with PBS (n = 3) or 20 μg of unconjugated scFv (n = 5). In contrast, mice that received tumors incubated with 20 μg of folate/scFv conjugate exhibited delayed growth of tumors (n = 4), or rejection of the tumor (n = 1). A recent report showed that folate levels in standard mouse chow are considerably higher then in human diet, leading to high serum folate levels (32). These levels could act to reduce the effectiveness of the FR-dependent treatments. For this reason, two mice that had been placed on a low folate diet also received KB cells mixed with the folate/scFv conjugate (1 μg). Neither of these mice developed tumors (Figure 6B). Future studies will evaluate the efficacy of the folate conjugates when different folate levels are provided in the diet.

In comparison to a subcutaneous model, an intraperitoneal tumor model more closely resembles advanced human ovarian cancer (51). Recent work has highlighted the improved efficacy of treating stage III ovarian cancer with intraperitoneal rather than intravenous cisplatin (52). We reasoned that a similar approach, using a large volume i.p. injection of folate/antibody conjugates might allow for improved delivery of antibody agents directly to the site of the tumor. In this experiment (Figure 7), each group of mice received 5 x 10^6 KB cells i.p. Similar to the s.c.

model, mice that received KB cells mixed with either PBS or 10 μg of unconjugated Fab fragments survived for equal times (median = 24 days and 23 days, respectively). In contrast, mice that were co-injected with 10 μg of either folate/Fab or folate/scFv survived for over 75 days, with two exceptions ($p < 0.004$). Statistical analyses of these and subsequent i.p. experimental groups are shown in Table 2.

Additional groups of mice were included to evaluate the effects of treating animals that did not receive a previous T cell activating agent (i.e. SIYRYYGL peptide)(n = 5) or to evaluate the effects of treating established tumors with i.p. injections of folate/Fab conjugates (n =5) or folate/scFv conjugates (n = 5). All mice that received KB cells together with the folate/Fab conjugate, but no SIYRYYGL peptide, exhibited delayed tumor growth although they eventually succumbed to the tumor (Figure 7 and Table 2). Thus, survival of mice that did not receive activating peptide was significantly reduced compared to the peptide-treated group ($p < 0.004$). These results show that prior T cell activation clearly enhances the anti-tumor effects of the folate conjugates.

Mice that received i.p. folate/antibody treatments of 2-day established tumors with either the folate/Fab or folate/scFv conjugates appeared to have increased survival times, although all of the mice eventually succumbed to the tumor (Figure 7 and Table 2). When analyzed as a treatment group receiving folate/antibody conjugates (n = 10), the treated animals exhibited a mean survival time = 38.8 days (SEM = 3.2) and a p value = 0.056.

Discussion

Many recent approaches to cancer therapy involve the induction of a patient's immune system against tumor cells (53, 54). Bispecific antibodies against a tumor antigen and an appropriate effector cell surface molecule provide one strategy toward achieving these effects (1, 2). Pre-clinical studies to evaluate bispecific antibodies that recognize human tumor antigens have required immunodeficient animal models. Typically in these models the human tumor, activated human effector cells, and the bispecific antibody to be evaluated are each administered to the

immunodeficient animal (i.e. nude or SCID mice). Unfortunately, these models do not accurately reflect the most desirable clinical uses of bispecific agents. These uses will include a treatment regimen in which only the antibody agents need be administered in order to redirect a patient's own immune system. In this report, we describe the development of a TCR/RAG-$1^{-/-}$ animal model that more resembles this clinical scenario and yet will allow anti-human tumor antigen reagents to be evaluated.

2C TCR transgenic mice were chosen for several reasons. First, these mice express a TCR that leads to the positive selection of T cells of the CD8 phenotype that are capable of cytolytic function (20). In addition, the 2C TCR transgenic system is particularly useful as there have been many studies to identify the peptides that are recognized by CTL 2C (23-25) and to explore the optimal activation requirements of 2C T cells (26-29). In principle, these peptides may be used to activate 2C transgenic T cells *in vivo*. As shown in Figure 3, the SIYRYYGL peptide can, in fact, activate cells in the TCR/RAG mice and these activated T cells are able to enhance the *in vivo* effects of the bispecific agents (Figure 7). Although this specific peptide will not be applicable to human therapy, it will now be possible to compare the general efficacy of peptide-induced activation with activation induced by other agents such as anti-CD3 and anti-CD28 antibodies. Improved anti-tumor effects might be achieved through the use of additional agents that can prolong the activity of T cells for longer times than the 1 to 2 days observed with SIYRYYGL peptide.

It is obvious that there are several factors that may lead to differences in the effectiveness of bispecific antibodies in tumor elimination, when comparing TCR/RAG mice with humans. For example, one might consider that TCR/RAG mice express a large population of homogenous $CD8^+$ cells that can act as cytolytic effectors. This population may prove to be more effective than is possible with individuals that express normal immune systems. On the other hand, these mice do not express $CD4^+$ cells that may enhance and sustain the anti-tumor effects when used in humans. That is, the monoclonal population of $CD8^+$ CTL precursor cells in the 2C TCR/RAG mice have achieved the entire anti-tumor effect acting in the absence of CD4-derived help. Furthermore, lysis

of human tumor cells by mouse CTLs is less efficient then the homocytotropic interaction because of interspecies differences in adhesion molecules ((48) and unpublished data). Finally, since the T cell repertoire of the 2C TCR/RAG mice is limited to a single clone, it is of course impossible to expect the expansion of tumor-specific T cells and a memory response upon rechallenge. With an intact immune system, it is possible that a redirected response against a syngeneic tumor could act to elicit tumor-specific T cells.

The issues raised above support the notion that bispecific antibodies should be tested in both xenograft system described here and in syngeneic mouse tumor models. In this respect, one of the key advantages of the folate conjugates described here is that the exact same conjugates can be compared in syngeneic mouse systems. Two different syngeneic tumors that express the folate receptor are available, the DBA/2 derived tumor F2-MTX^{r}A (41) and choroid plexus tumors that arise endogenously in an SV40 transgenic mouse line (55).

The use of the 2C TCR/RAG system also will allow the evaluation of aspects of T cell trafficking in mice. These studies can be conveniently performed because all of the T cells are monoclonal and therefore express the same TCR identifiable by the anti-clonotypic antibody 1B2 (48). Thus, it will be possible to explore the optimal activating and retargeting agents as well as the regimen required to maximize infiltration of T cells into the tumor. This will be particularly important as our findings have shown that treatment of established solid KB tumors with folate conjugates will require improvements in this aspect of the therapy. Other studies have found similar problems associated with bispecific antibody therapy of solid tumors (e.g. (16, 51)). The 2C TCR/RAG mice provide a well-defined, monoclonal system in which the effects of various T cell-specific agents can be evaluated without complications inherent in polyclonal lymphoid systems.

In order to exploit the advantages of the TCR/RAG system, we have developed a human tumor model that resembles FR^{+} ovarian carcinomas. In the present studies the human tumor line KB, that has been used for many *in vitro* studies with folate receptor-specific agents, was injected either s.c. or i.p. The i.p. route of administration of the KB tumor line is similar to disseminated

human ovarian tumors in that most of the tumor remains within the peritoneal cavity. Mice with established i.p. tumors did exhibit an approximate doubling of survival times after treatment with folate/anti-TCR conjugates. Our recent findings using the endogenous brain tumor model in SV40 transgenic mice also showed that folate/scFv treatment could extend survival approximate two times longer then control mice, after initial signs of tumor development. Thus, it will be important to identify agents which can improve the penetration of bispecific antibodies and effector cells into poorly-vascularized, non-hematopoetic solid tumors. A more complete understanding of T cell activation and migration properties (56) should allow the identification of agents that have improved T cell modulating properties.

This study also demonstrates the significant therapeutic potential of a new class of targeting agents that are smaller and easier to produce then conventional bispecific antibodies. These folate/antibody conjugates are capable of targeting tumors that bear the high affinity folate receptor at low doses (41, 42). In addition, the ease of producing them will allow the study of other folate/antibody conjugates (e.g. folate/anti-CD28) to test their ability to enhance T cell responses in the TCR/RAG system decribed here. The effectiveness of the folate conjugate treatments in mice is likely reduced somewhat by the high folate levels that are found in normal rodent chow, and thus in the sera of mice (32). Imaging with folate conjugates has been improved by lowering the circulating levels of folate in mice to human serum levels by feeding mice low folate chow (32). Based on these results, it is reasonable to predict that folate/anti-T cell conjugate treatments may be more effective if the TCR/RAG mice are maintained on low-folate diets.

Finally, it is notable that other tumors, such as the human erbB-2$^+$ tumors BT474 and SKOV3 also readily grow in the TCR/RAG mice. Thus, it will be possible to evaluate other bispecific anti-human tumor antigen antibodies in the TCR/RAG system. In the case of the KB line, we have found a 100% tumor acceptance rate in TCR/RAG mice, providing further evidence that these mice do not exhibit the "leakiness" of SCID mice. Our ultimate goal is to develop a model in which combinations of agents to be used in humans could be tested against human tumors transplanted into animals that, like the patient, contain competent endogenous immune effector

cells. The TCR/RAG mice described here approach this goal, although the anti-effector agent remains an anti-mouse T cell receptor antibody. In the future, this aspect can be improved by either using antibodies that cross-react with human and mouse antigen-receptors, or possibly by the devlopment of transgenic mice that express the relevant human antigen (e.g. human CD3).

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Table 1. Effect of antibody treatments on subcutaneous KB tumor growth.

Treatment	n	Median[a] days	Mean[a] + SEM days	P Values[b]
Experiment 1				
none	3	38	36.0±2.5	-
folate/Fab (20 μg)	3	>114	>107.3[c]	0.03
Experiment 2				
none	3	35	37.3±2.3	-
scFv	5	35	34.8±1.8	0.44
folate/scFv (20μg)	5	70	70.6±9.8	0.05
folate/scFv (1μg)	2	88	>117[d]	0.05

[a]Number of days taken to reach a mean tumor diameter of 20mm.

[b] Comparison of each treatment to the KB control, untreated group.

[c] One mouse had a tumor reach 20 mm at 94 days while 2 mice had no visible tumor on day 114 at which time they were sacrificed.

[d]One mouse died at 59 days, apparently of a non-tumor related cause, while the second mouse shows no sign of a tumor at 117 days.

Table 2. Effect of antibody treatments on survival of mice that received intraperitoneal KB tumors.

Treatment	n	Median	Mean + SEM	P Values[a]
none	5	24	29.2±4.5	-
Fab	5	23	23.6±1.2	0.08
folate/Fab	5	>70[b]	>70	<0.004
folate/scFv	5	>70[c]	>70	<0.004
folate/Fab (without peptide)	5	47	47.6±2.0	0.03
folate/Fab (established)	5	44	40.0±4.8	0.07
folate/scFv (established)	5	45	37.6±4.5	0.23

[a] Comparison of each treatment to the KB control, untreated group.

[b] At the time of submission (day 70) all mice were alive in this treatment group.

[c] At the time of submission (day 70) 4 mice were alive and one died on day 55 apparently of a non-tumor related cause.

Figure Legends

Figure 1. Tumor growth of KB xenografts in RAG and TCR/RAG mice. **A)** KB cells ($1x10^7$ in 200µl) were injected s.c. into RAG (○) and TCR/RAG (■) mice. **B)** Growth of s.c. xenografts of $1x10^7$ (■) or $3x10^6$ (□) cells in TCR/RAG mice. Vernier calipers were used to take 2 perpendicular tumor measurements twice weekly. Measurements were averaged to determine mean tumor diameter.

Figure 2. Immunohistochemistry of folate receptor in KB and RMAS tumor cells after growth in TCR/RAG mice. Subcutaneous tumors of the human FR-postive line KB **(A-C)** and the mouse FR-negative line RMAS **(D-F)** were examined by immunohistochemistry. In (A) and (D) tumors were stained with H&E. In (B) and (E), sections were incubated with a primary rabbit anti-folate receptor antiserum (obtained from Dr. Kevin Brigle) followed by a biotinylated secondary anti-rabbit Ig, avidin-biotin HRP, and DAB to generate brown reactive product. In (C) and (F), primary antibody was omitted as a control. Only the KB tumor had detectable FR immunoreactivity (B).

Figure 3. Ex vivo cytolytic activty of TCR/RAG splenocytes following treatment with SIYRYYGL peptide. **(A)** Splenocytes were assayed after 1(▲), 2 (■), or 3 (□) days of a single 10 nmol i.p. injection of SIYRYYGL peptide (1 x 10 nmol); or 1 day after a second injection of 10 nmol peptide injections on consecutive days (◪) (2 x 10 nmol). **(B)** Similar results were observed with a 2.5 nmol dose of peptide: 1 day after a single injection (○)(1 x 2.5 nmol) or 1 day after a second injection (●) (2 x 2.5 nmol). Splenocytes were assayed at various E:T ratios against ^{51}Cr labeled P815 target cells. Standard error bars represent the variation of triplicate wells within the same assay. No cytolytic activity was observed with splenocytes from mice that did not receive peptide (data not shown).

Figure 4. Tumor growth curves of KB xenografts in TCR/RAG mice treated with SIYRYYGL peptide. Mice treated one day previously with 2.5 nmol SIYRYYGL peptide (□, n=3) or without SIYRYYGL peptide (●, n=2), were injected s.c. with KB cells ($1x10^7$ in 200µl). Vernier calipers were used to take 2 perpendicular tumor measurements twice weekly. Measurements were averaged to determine mean tumor diameter.

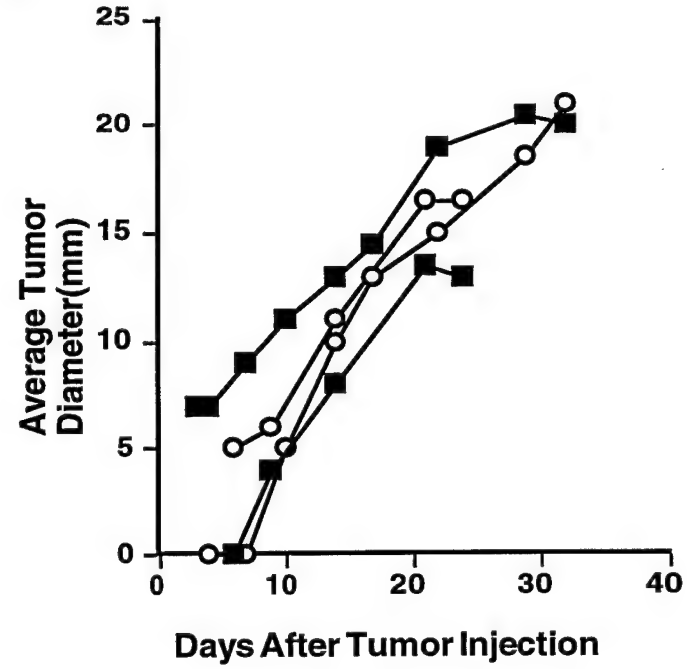
Figure 5. In vitro, CTL-mediated lysis of KB tumor cells in the presence of folate/anti-TCR conjugates. **(A)** Specific lysis of human KB cells by the mouse CTL clone 2C mediated by KJ16 folate conjugates. The mouse CTL clone 2C was used as the effector and the FR positive human KB cell line as the ^{51}Cr-labeled target ($1 x10^4$cells) at an effector to target (E:T) cell ratio of 67:1. Various concentrations of folate/ KJ16 Fab (□) and folate/scFv (■) were added to the cells in triplicate wells in folate free DMEM + 10% FCS. Excess free folate (1.2 µM) was added to the highest antibody concentration of each conjugate (folate/KJ16 Fab (○), folate/KJ16 scFv (▲)). **(B)** Specific lysis of human KB cells by activated TCR/RAG splenocytes mediated by KJ16 folate conjugates. *In vitro* activated TCR/RAG splenocytes were prepared by incubation of spleen cells with 10 nM SIYRYYGL and 10% supernatant from concanavalin A-treated rat splenocytes for 3 days in culture. Activated TCR/RAG splenocytes were used as the effector and the FR positive human KB cell line as the ^{51}Cr-labeled target ($5 x10^3$cells) at an effector to target (E:T) cell ratio of 18:1. Various concentrations of folate/ KJ16 Fab (□) and folate/scFv (■) were added to the cells in triplicate wells in folate free DMEM + 10% FCS. Specific ^{51}Cr release was calculated as described in *Materials and Methods.*

Figure 6. Effects of folate/antibody conjugates on the subcutaneous growth of KB cells. (A) Experiment 1. KB cells (3 x10^6) mixed with PBS (●) or 20μg folate/KJ16 Fab fragments (□) or were injected subcutaneously into TCR/RAG mice, 24 hours following SIYRYYGL peptide administration (2.5 nmol i.p.). Mice received a second peptide dose on day 3 an a second antibody treatment (20 μg; i.p.) on day 4 following tumor injection. (B) Experiment 2. KB cells (2.6x10^6) mixed with PBS (○), 20 μg folate/scFv-KJ16 (▲), 1 μg folate/scFv-KJ16 (□), or 20 μg scFv-KJ16 (●) were injected subcutaneously into TCR/RAG mice, 24 hours following SIYRYYGL peptide administration (2.5 nmol i.p.). The treatment regime was identical to Experiment 1 except that mice which received the 1μg folate/scFv treatment were placed on low folate chow 3 weeks prior to the tumor injection. *This mouse died on day 59 apparently of a non-tumor related cause. Tumor measurements were made twice weekly and mice were euthanized when their tumor reached a mean diameter of 20 mm. Mean tumor diameter was the average of 2 perpendicular measurements.

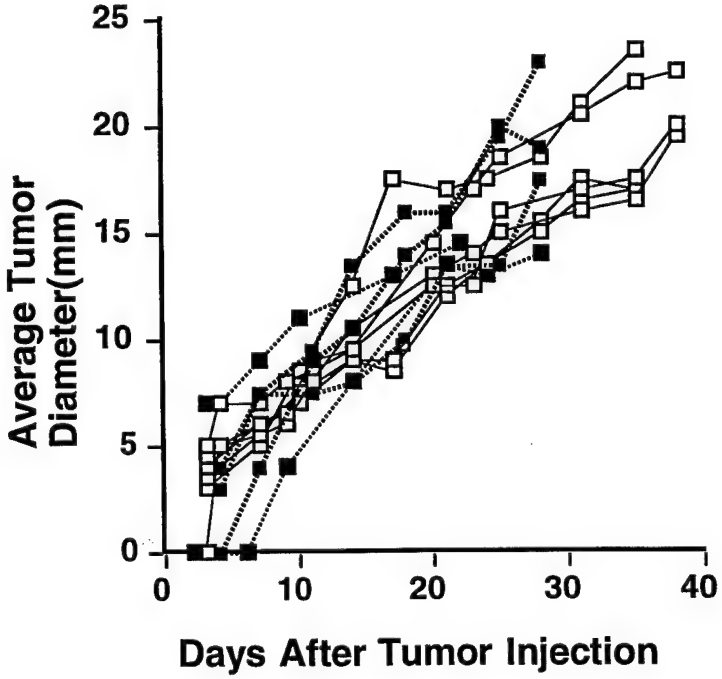
Figure 7. Effects of folate/antibody conjugates on the survival of TCR/RAG mice with peritoneal KB tumors. KB cells (5x10^6) mixed with PBS (○), 10 μg KJ16 Fab fragments (●), 10μg folate/KJ16 Fab fragments (■), or 10μg folate/scFv (□) were injected i.p. into TCR/RAG mice (n = 5 per treatment) as a single cell suspension in 1 ml. Antibodies were administered twice, mixed with the cells and 4 days later. For the established groups, antibodies wee added on days 2 and 6 after tumor injection (established folate/Fab fragments ◆; established folate/scFv ◇). In all except the NO SIYRYYGL treatment group (△), 2.5 nmol of SIYRYYGL peptide was administered i.p. 24 hr. prior to each antibody injection. The NO SIYRYYGL treatment group (△) received 10μg folate/KJ16 Fab fragments mixed with KB cells as in

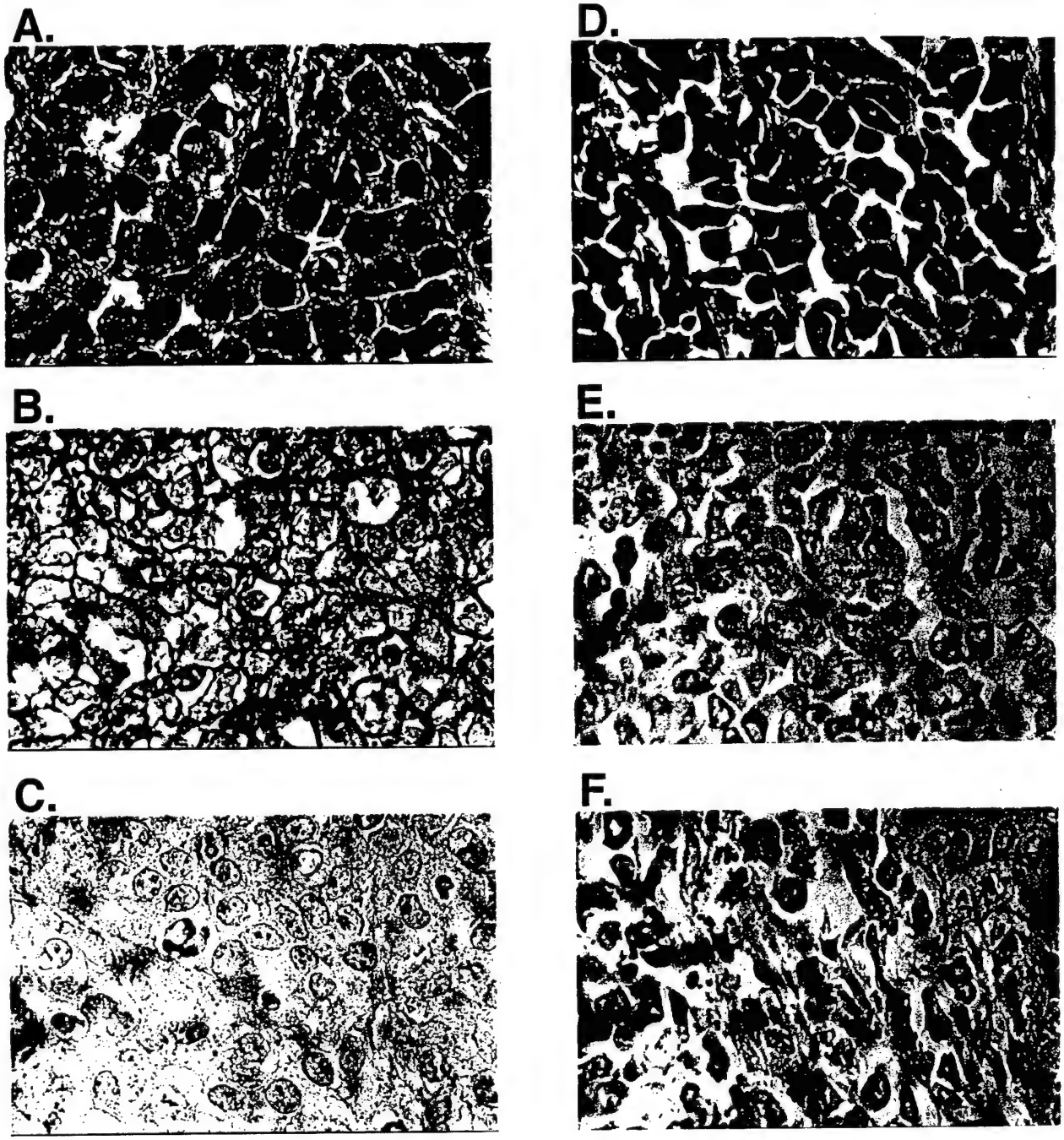
experimental group (■). Mice were monitored daily for signs of illness and were euthanized as they became moribund. Results for the mouse with an * died on day 55 apparently of a non-tumor related cause. The time line indicates the treatment regime used in the experiment: Mixed refers to animals that received a mixture of the antibody and tumor at the time of transplantation (day 0); Established refers animals that received only tumor on day 0.

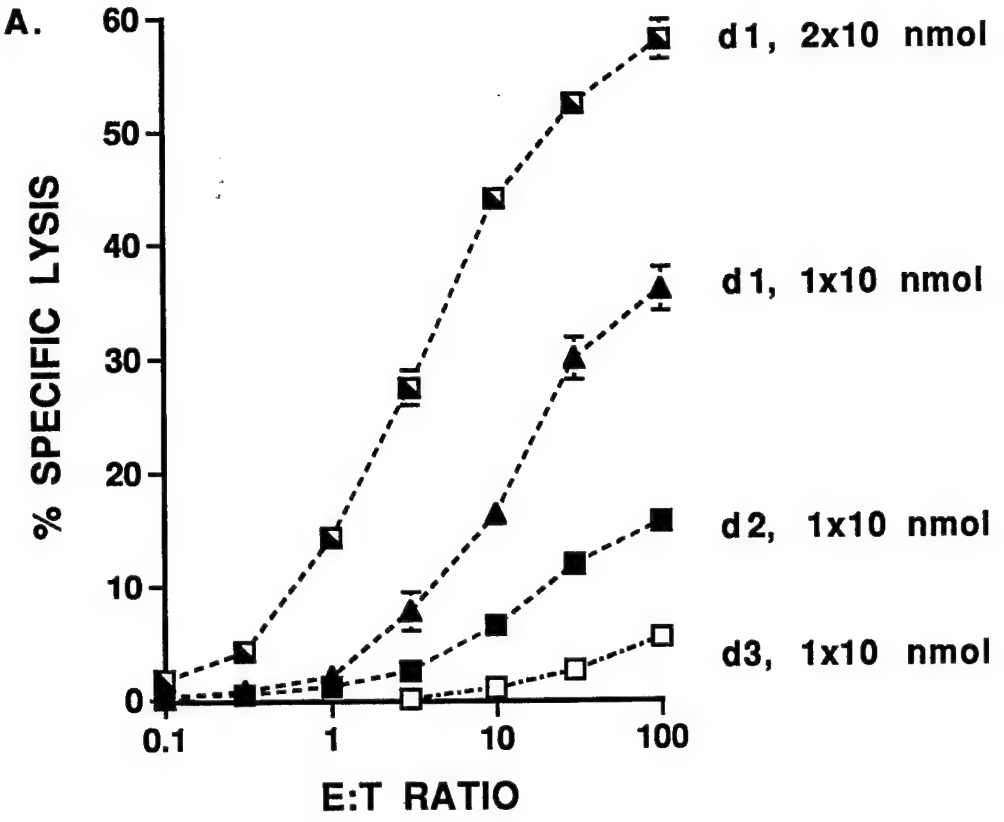
A.

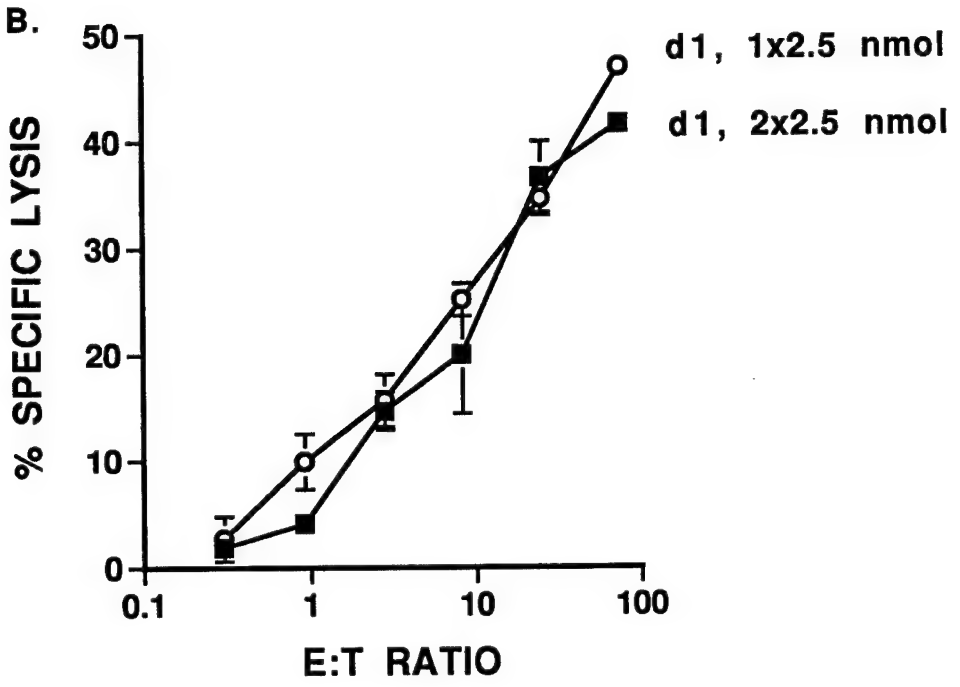

25
20
15
10
5
0
0
10
20
30
40
Average Tumor Diameter(mm)
Days After Tumor Injection

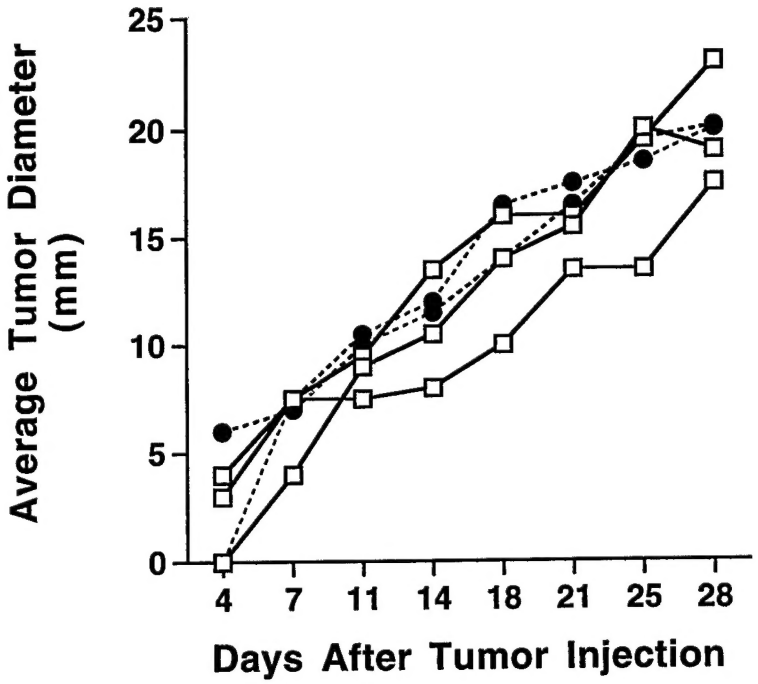
B.


25
20
15
10
5
0
0
10
20
30
40
Average Tumor Diameter(mm)
Days After Tumor Injection

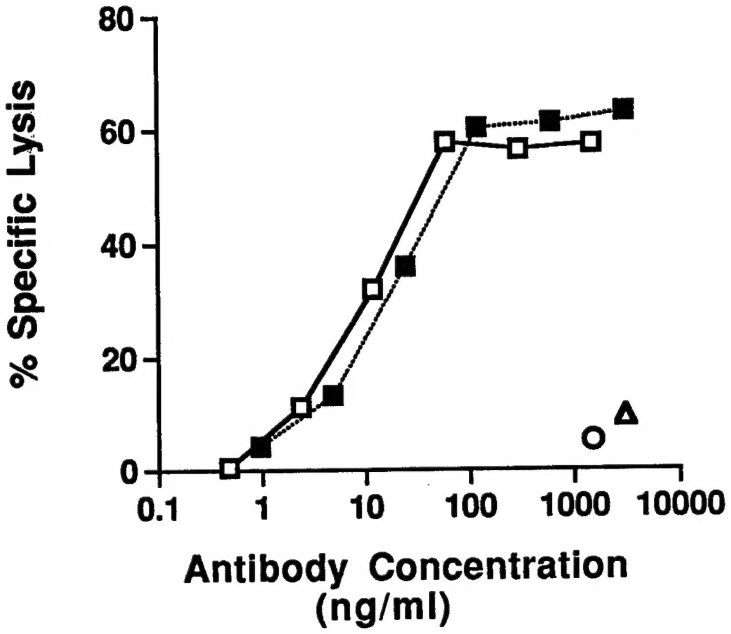

A.
D.
B.
E.
C.
F.


A.
% SPECIFIC LYSIS
60
50
40
30
20
10
0
0.1
1
10
100
E:T RATIO
d1, 2x10 nmol
d1, 1x10 nmol
d2, 1x10 nmol
d3, 1x10 nmol

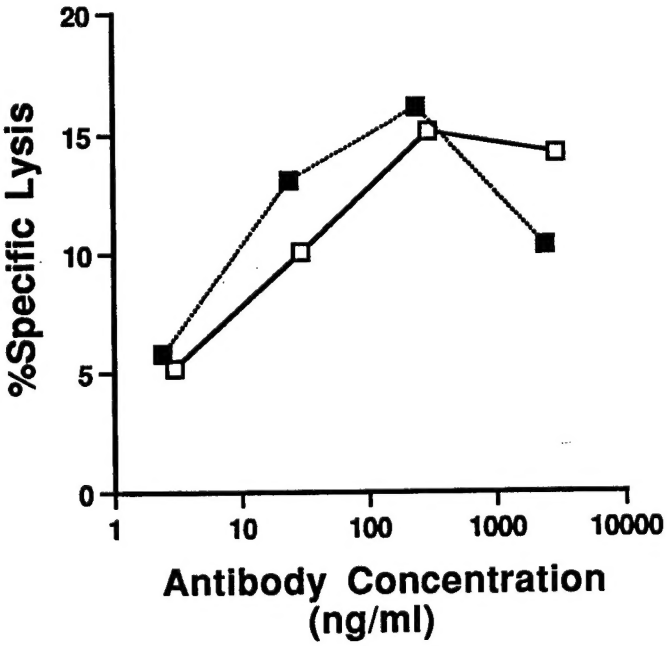

B.
% SPECIFIC LYSIS
50
40
30
20
10
0
0.1
1
10
100
E:T RATIO
d1, 1x2.5 nmol
d1, 2x2.5 nmol


Average Tumor Diameter (mm)
25
20
15
10
5
0
4
7
11
14
18
21
25
28
Days After Tumor Injection

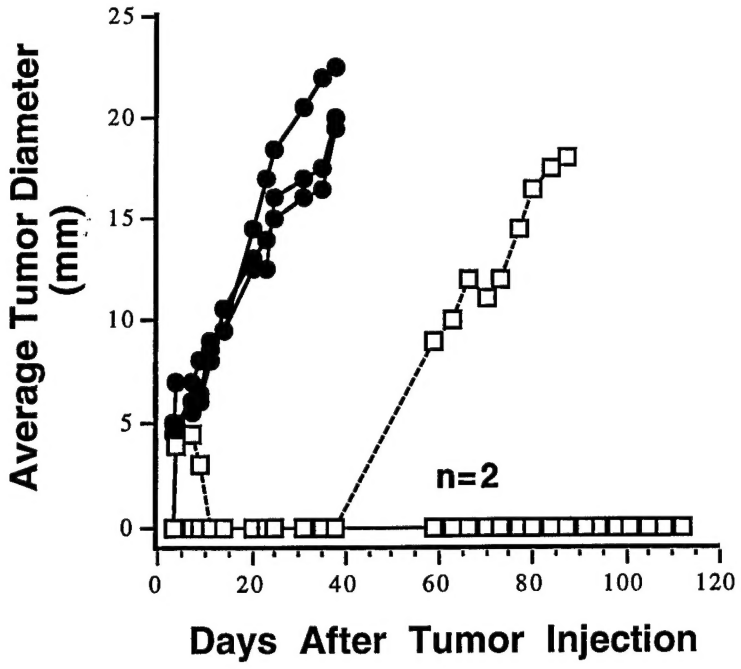
A.


80
60
40
20
0
% Specific Lysis
0.1
1
10
100
1000
10000
Antibody Concentration
(ng/ml)

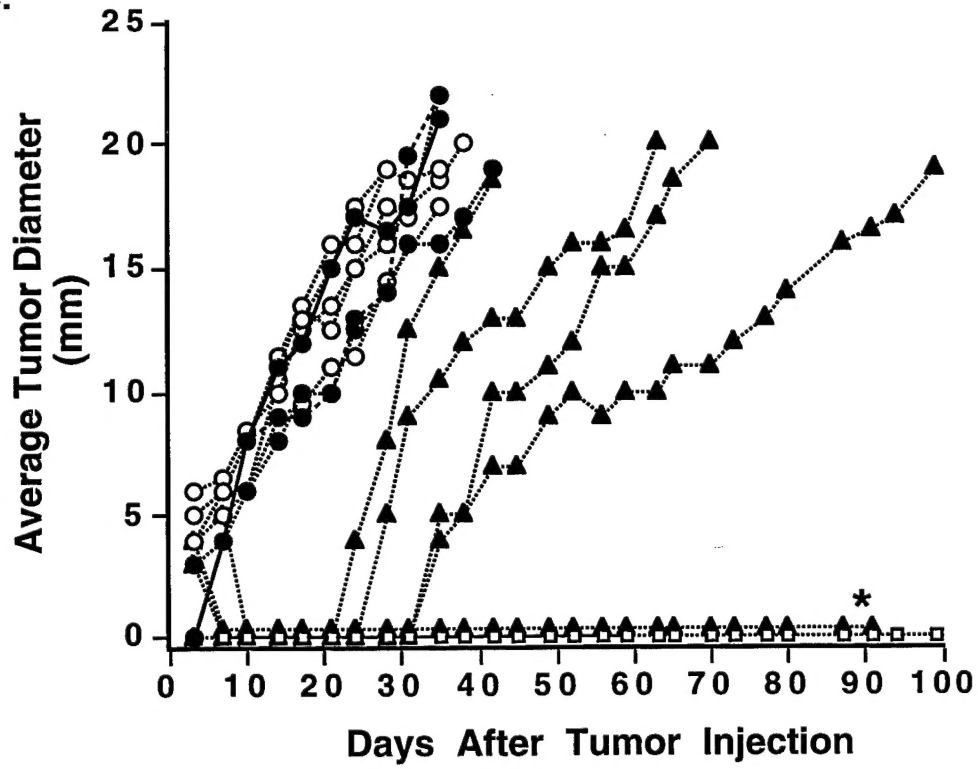
B.

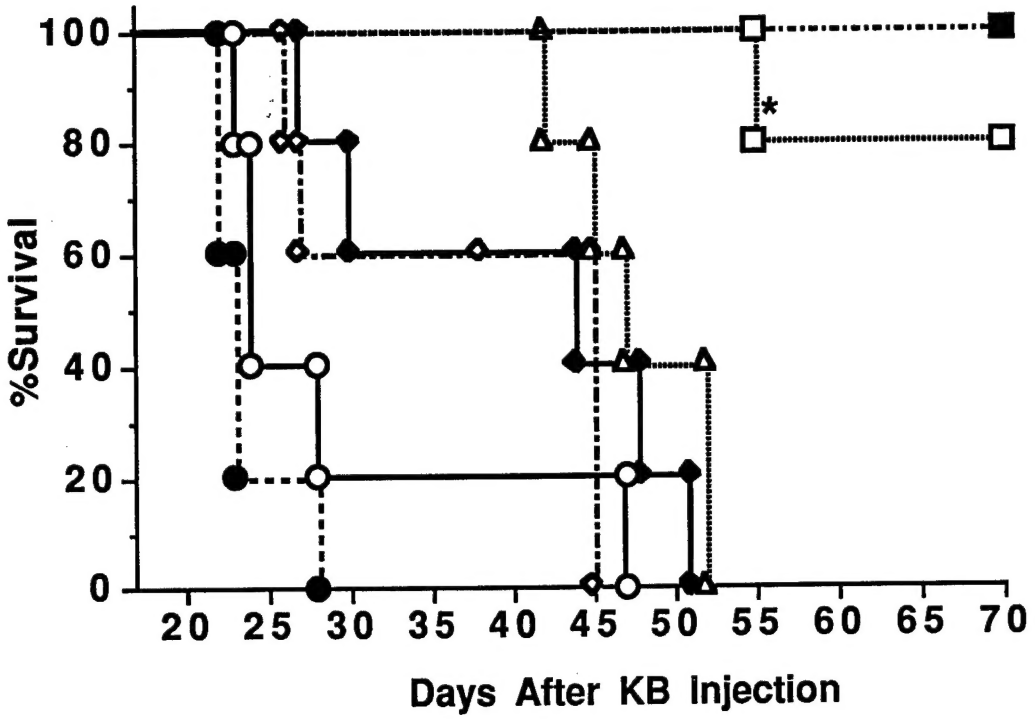

20
15
10
5
0
%Specific Lysis
1
10
100
1000
10000
Antibody Concentration
(ng/ml)

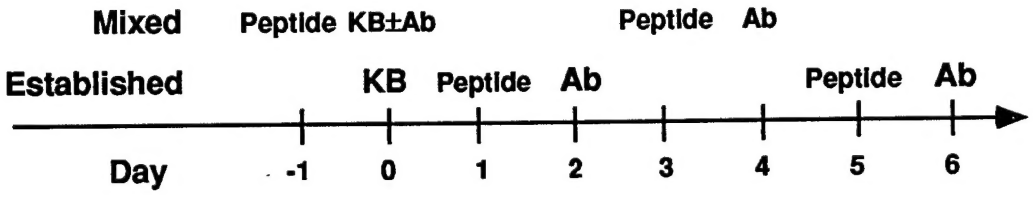
A.


Average Tumor Diameter (mm)
25
20
15
10
5
0
n=2
0
20
40
60
80
100
120
Days After Tumor Injection

B.


Average Tumor Diameter (mm)
25
20
15
10
5
0
*
0
10
20
30
40
50
60
70
80
90
100
Days After Tumor Injection


100
80
60
40
20
0
%Survival
20 25 30 35 40 45 50 55 60 65 70
Days After KB Injection
*


Mixed
Peptide KB±Ab
Peptide Ab
Established
KB Peptide Ab
Peptide Ab
Day
-1 0 1 2 3 4 5 6